

PATENT APPLICATION

INTEGRATED NUCLEIC ACID DIAGNOSTIC DEVICE

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5 INTEGRATED NUCLEIC ACID DIAGNOSTIC DEVICE

This application is a continuation of U.S. Patent Application 09/294,700, filed on April 19, 1999, now U.S. Patent _____; which is a divisional of U.S. Patent Application 08/671,928, filed on June 27, 1996, now U.S. Patent 5,922,591, which is a continuation-in-part of U.S. Patent Application 10 08/589,027, filed on January 19, 1996, now U.S. Patent 5,856,174, which claims priority from U.S. Provisional Application 60/000,703, filed on June 29, 1995 and also claims priority from U.S. Provisional Application 60/000,859, filed on July 3, 1995. This application is also a continuation-in-part of U.S. Patent Application 09/210,025, filed on December 11, 1998 now U.S. Patent 6,043,080. The above-identified 15 applications are incorporated herein by reference in its entirety for all purposes.

GOVERNMENT RIGHTS

The present invention was made with U.S Government support under ATP Grant No. 70NANB5H1031. The government has 20 certain rights in this invention.

BACKGROUND OF THE INVENTION

The relationship between structure and function of macromolecules is of fundamental importance in the 25 understanding of biological systems. These relationships are important to understanding, for example, the functions of enzymes, structure of signalling proteins, ways in which cells communicate with each other, as well as mechanisms of cellular control and metabolic feedback.

30 Genetic information is critical in continuation of life processes. Life is substantially informationally based and its genetic content controls the growth and reproduction of the organism. The amino acid sequences of polypeptides, which are critical features of all living systems, are encoded 35 by the genetic material of the cell. Further, the properties of these polypeptides, e.g., as enzymes, functional proteins, and structural proteins, are determined by the sequence of amino acids which make them up. As structure and function are

integrally related, many biological functions may be explained by elucidating the underlying structural features which provide those functions, and these structures are determined by the underlying genetic information in the form of 5 polynucleotide sequences. In addition to encoding polypeptides, polynucleotide sequences can also be specifically involved in, for example, the control and regulation of gene expression.

The study of this genetic information has proved to 10 be of great value in providing a better understanding of life processes, as well as diagnosing and treating a large number of disorders. In particular, disorders which are caused by mutations, deletions or repeats in specific portions of the genome, may be readily diagnosed and/or treated using genetic 15 techniques. Similarly, disorders caused by external agents may be diagnosed by detecting the presence of genetic material which is unique to the external agent, e.g., bacterial or viral DNA.

While current genetic methods are generally capable 20 of identifying these genetic sequences, such methods generally rely on a multiplicity of distinct processes to elucidate the nucleic acid sequences, with each process introducing a potential for error into the overall process. These processes also draw from a large number of distinct disciplines, 25 including chemistry, molecular biology, medicine and others. It would therefore be desirable to integrate the various process used in genetic diagnosis, in a single process, at a minimum cost, and with a maximum ease of operation.

Interest has been growing in the fabrication of 30 microfluidic devices. Typically, advances in the semiconductor manufacturing arts have been translated to the fabrication of micromechanical structures, e.g., micropumps, microvalves and the like, and microfluidic devices including miniature chambers and flow passages.

35 A number of researchers have attempted employ these microfabrication techniques in the miniaturization of some of the processes involved in genetic analysis in particular. For

example, published PCT Application No. WO 94/05414, to Northrup and White, incorporated herein by reference in its entirety for all purposes, reports an integrated micro-PCR apparatus for collection and amplification of nucleic acids from a specimen. However, there remains a need for an apparatus which combines the various processing and analytical operations involved in nucleic acid analysis. The present invention meets these and other needs.

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SUMMARY OF THE INVENTION

The present invention generally provides miniature integrated fluidic systems for carrying out a variety of preparative and analytical operations, as well as methods of operating these systems and methods of using these systems. In 15 a first aspect, the present invention provides a miniature fluidic system which comprises a body having at least first and second chambers disposed therein. Each of these first and second chambers has a fluid inlet and is in fluid connection. At least one of these first and second chambers is a 20 hybridization chamber for analyzing a component of a fluid sample. The hybridization chamber includes a polymer array which has a plurality of different polymer sequences coupled to a surface of a single substrate, each of the plurality of different polymer sequences being coupled to the surface in a 25 different, known location. The system further includes a sample inlet, fluidly connected to at least one of the first and second chambers, for introducing a fluid sample into the system, and a fluid transport system for moving a fluid sample from the first chamber to the second chamber.

30 In a preferred aspect, the fluid direction system comprises a pneumatic manifold for applying a differential pressure between the first chamber and the second chamber, to move said fluid sample from the first chamber to the second chamber.

35 In a related aspect, the present invention provides a miniature fluidic system, which is substantially the same as that described above, except that in place or in addition to a

hybridization chamber, the system comprises a separation channel for separating a component of said fluid sample. The separation channel is fluidly connected to at least one of the chambers and includes at least first and second electrodes in 5 electrical contact with opposite ends of the separation channel for applying a voltage across said separation channel.

Similarly, in an additional aspect, the present invention provides a substantially similar fluidic system as described, except where at least one of the chambers comprises 10 an in vitro transcription reaction chamber, the in vitro transcription reaction chamber having an effective amount of an RNA polymerase and four different nucleoside triphosphates, disposed therein.

Further, the system may comprise a body wherein at 15 least one of the chambers is a cell lysis chamber which includes a cell lysis system, for lysing cells in said fluid sample.

In a still further related aspect, at least one of the chambers may be a nucleic acid purification chamber, for 20 separating nucleic acids in said fluid sample from other contaminants in said fluid sample.

The present invention also provides a miniature fluidic system which comprises a differential pressure delivery system for transporting fluids through the system.

25 In particular, in one aspect, the present invention provides a miniature fluidic system, which includes a body having at least a first reaction chamber fluidly connected to a second reaction chamber by a fluid passage. The system also includes a sample inlet, fluidly connected to the first chamber, for 30 introducing a fluid sample into the system. The system further includes a differential pressure delivery system for maintaining the first chamber at a first pressure and the second chamber at a second pressure, wherein the first pressure is greater than ambient pressure and the second 35 pressure is greater than said first pressure. When the second chamber is brought to ambient pressure, the first pressure

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forces a liquid sample in the first chamber into the second chamber.

In an alternate aspect, the fluidic system employs a differential pressure delivery source for maintaining the 5 first chamber at a first pressure and the second chamber at a second pressure, where the second pressure is less than ambient pressure and the first pressure is less than the second pressure. When the first chamber is brought to ambient pressure, the second pressure draws a liquid sample in the 10 first chamber into the second chamber.

The present invention also provides methods of directing, controlling and manipulating fluids in miniature or micro-fluidic systems.

For example, in one aspect, the present invention 15 provides a method for directing a fluid sample in a miniature fluidic system which comprises providing a microfabricated device having at least first and second chambers disposed therein, wherein each of said at least first and second chambers is in fluid connection with a common chamber or 20 channel, has at least first and second controllable valves disposed across said fluid connection, respectively, and includes at least one vent. The method comprises applying a positive pressure to the common chamber or channel. The at least first controllable valve is selectively opened, whereby 25 the positive pressure forces the fluid sample from the common chamber or channel into the first chamber.

The method may further comprise applying a positive pressure to the first chamber and selectively opening the least first controllable valve, whereby the positive pressure 30 forces said fluid sample from the least first chamber into the common chamber or channel.

The present invention also provides methods of mixing at least two discrete fluid components in a microfabricated fluidic system. Specifically, the method 35 comprises providing a microfabricated channel having a vent disposed at an intermediate location in said channel. Typically, the vent includes a gas permeable, fluid barrier

disposed across the vent. At least two discrete fluid components are then introduced into the channel separated by a gas bubble. Upon flowing the at least two fluid components past the vent, the bubble will exit the vent, allowing the at 5 least two fluid components to mix.

The present invention also provides methods of repeatedly measuring a known volume of a fluid in a miniature fluidic system. In particular, the method comprises providing a microfabricated device having at least first and second 10 chambers disposed therein, wherein the at least first and second chambers are in fluid connection, and wherein at least one of the chambers is a volumetric chamber having a known volume. The volumetric chamber is filled with the fluid to create a first aliquot of the fluid. This aliquot is then 15 transported to the at least second chamber and the filling and transporting steps are repeated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of a 20 nucleic acid diagnostic system for analysis of nucleic acids from samples.

Figures 2A and 2b show schematic representations of two alternate reaction chamber designs from a cut-away view.

Figure 3 shows a schematic representation of a 25 miniature integrated diagnostic device having a number of reaction chambers arranged in a serial geometry.

Figures 4A-C show a representation of a microcapillary electrophoresis device. Figures 4A and 4B show the microcapillary configured for carrying out alternate 30 loading strategies for the microcapillary whereas Figure 4C illustrates the microcapillary in running mode.

Figure 5A illustrates a top view of a miniature integrated device which employs a centralized geometry. Figure 5B shows a side view of the same device wherein the 35 central chamber is a pumping chamber, and employing diaphragm valve structures for sealing reaction chambers.

Figure 6 shows schematic illustrations of pneumatic control manifolds for transporting fluid within a miniature integrated device. Figure 6A shows a manifold configuration suitable for application of negative pressure, or vacuum, 5 whereas Figure 6B shows a manifold configuration for application of positive pressures. Figure 6C illustrates a pressure profile for moving fluids among several reaction chambers.

Figure 7A shows a schematic illustration of a 10 reaction chamber incorporating a PZT element for use in mixing the contents of the reaction chamber. Figure 7B shows mixing within a reaction chamber applying the PZT mixing element as shown in Figure 7A. Figure 7C is a bar graph showing a 15 comparison of hybridization intensities using mechanical mixing, acoustic mixing, stagnant hybridization and optimized acoustic mixing.

Figure 8 is a schematic illustration of a side and top view of a base-unit for use with a miniature integrated device.

20 Figure 9 is a time temperature profile of thermal cycling in a miniature reaction chamber and a display of the programmed cycling parameters.

Figure 10A is a gel showing a time course of an RNA 25 fragmentation reaction. Figure 10B is a gel showing a comparison of the product of an in vitro transcription reaction in a microchamber vs. a control (test tube). Figure 10C is a comparison of the PCR product produced in a PCR thermal cycler and that produced by a microreactor.

Figure 11 shows an embodiment of a reaction chamber 30 employing an electronic pH control system.

Figure 12A-C show a schematic representation of a miniature integrated device employing a pneumatic fluid direction system utilizing a gas permeable fluid barrier bound vents, e.g., a poorly wetting or hydrophobic membrane, and 35 pneumatically controlled valves. Figure 12A shows an embodiment of a single chamber employing this system. Figure 12B is a schematic illustration of a debubbling chamber for

linking discrete fluid plugs that are separated by a gas bubble. Figure 12C schematically illustrates this system in an integrated device having numerous chambers, including degassing chamber, dosing or volumetric chamber, storage and 5 reaction chambers. Figure 12D is an illustration of an injection molded substrate which embodies the system schematically illustrated in Figure 12C.

Figure 13 is a schematic representation of a device configuration for carrying generic sample preparation 10 reactions.

Figure 14 is a schematic representation of a device configuration for carrying multiple parallel reactions.

Figure 15 shows a demonstration of integrated reactions in a microfabricated polycarbonate device. Figure 15A shows the layout of the device including the thermal configuration of the device. Figure 15B shows the results of PCR amplification and subsequent in vitro transcription within the chambers of the device.

20 DETAILED DESCRIPTION OF THE INVENTION

I. General

It is a general object of the present invention to provide a miniaturized integrated nucleic acid diagnostic devices and systems incorporating these devices. The device 25 of the invention is generally capable of performing one or more sample acquisition and preparation operations, in combination with one or more sample analysis operations. For example, the device can integrate several or all of the operations involved in sample acquisition and storage, sample 30 preparation and sample analysis, within a single, miniaturized, integrated unit. The device is useful in a variety of applications and most notably, nucleic acid based diagnostic applications and *de novo* sequencing applications.

The device of the invention will typically be one 35 component of a larger diagnostic system which further includes a reader device for scanning and obtaining the data from the device, and a computer based interface for controlling the

device and/or interpretation of the data derived from the device.

To carry out its primary function, one embodiment of the device of the invention will typically incorporate a plurality of distinct reaction chambers for carrying out the sample acquisition, preparation and analysis operations. In particular, a sample to be analyzed is introduced into the device whereupon it will be delivered to one of these distinct reaction chambers which are designed for carrying out a variety of reactions as a prelude to analysis of the sample. These preparative reactions generally include, e.g., sample extraction, PCR amplification, nucleic acid fragmentation and labeling, extension reactions, transcription reactions and the like.

Following sample preparation, the sample can be subjected to one or more different analysis operations. A variety of analysis operations may generally be performed, including size based analysis using, e.g., microcapillary electrophoresis, and/or sequence based analysis using, e.g., hybridization to an oligonucleotide array. In addition to the various reaction chambers, the device will generally comprise a series of fluid channels which allow for the transportation of the sample or a portion thereof, among the various reaction chambers. Further chambers and components may also be included to provide reagents, buffers, sample manipulation, e.g., mixing, pumping, fluid direction (i.e., valves) heating and the like.

II. Integratable Operations

30 A. Sample Acquisition

The sample collection portion of the device of the present invention generally provides for the identification of the sample, while preventing contamination of the sample by external elements, or contamination of the environment by the sample. Generally, this is carried out by introducing a sample for analysis, e.g., preamplified sample, tissue, blood, saliva, etc., directly into a sample collection chamber within

the device. Typically, the prevention of cross-contamination of the sample may be accomplished by directly injecting the sample into the sample collection chamber through a sealable opening, e.g., an injection valve, or a septum. Generally, 5 sealable valves are preferred to reduce any potential threat of leakage during or after sample injection. Alternatively, the device may be provided with a hypodermic needle integrated within the device and connected to the sample collection chamber, for direct acquisition of the sample into the sample 10 chamber. This can substantially reduce the opportunity for contamination of the sample.

In addition to the foregoing, the sample collection portion of the device may also include reagents and/or treatments for neutralization of infectious agents, 15 stabilization of the specimen or sample, pH adjustments, and the like. Stabilization and pH adjustment treatments may include, e.g., introduction of heparin to prevent clotting of blood samples, addition of buffering agents, addition of protease or nuclease inhibitors, preservatives and the like. 20 Such reagents may generally be stored within the sample collection chamber of the device or may be stored within a separately accessible chamber, wherein the reagents may be added to or mixed with the sample upon introduction of the sample into the device. These reagents may be incorporated 25 within the device in either liquid or lyophilized form, depending upon the nature and stability of the particular reagent used.

B. Sample Preparation

In between introducing the sample to be analyzed 30 into the device, and analyzing that sample, e.g., on an oligonucleotide array, it will often be desirable to perform one or more sample preparation operations upon the sample. Typically, these sample preparation operations will include such manipulations as extraction of intracellular material, 35 e.g., nucleic acids from whole cell samples, viruses and the like, amplification of nucleic acids, fragmentation, transcription, labeling and/or extension reactions. One or

more of these various operations may be readily incorporated into the device of the present invention.

C. DNA Extraction

For those embodiments where whole cells, viruses or other tissue samples are being analyzed, it will typically be necessary to extract the nucleic acids from the cells or viruses, prior to continuing with the various sample preparation operations. Accordingly, following sample collection, nucleic acids may be liberated from the collected cells, viral coat, etc., into a crude extract, followed by additional treatments to prepare the sample for subsequent operations, e.g., denaturation of contaminating (DNA binding) proteins, purification, filtration, desalting, and the like.

Liberation of nucleic acids from the sample cells or viruses, and denaturation of DNA binding proteins may generally be performed by physical or chemical methods. For example, chemical methods generally employ lysing agents to disrupt the cells and extract the nucleic acids from the cells, followed by treatment of the extract with chaotropic salts such as guanidinium isothiocyanate or urea to denature any contaminating and potentially interfering proteins. Generally, where chemical extraction and/or denaturation methods are used, the appropriate reagents may be incorporated within the extraction chamber, a separate accessible chamber or externally introduced.

Alternatively, physical methods may be used to extract the nucleic acids and denature DNA binding proteins. U.S. Patent No. 5,304,487, incorporated herein by reference in its entirety for all purposes, discusses the use of physical protrusions within microchannels or sharp edged particles within a chamber or channel to pierce cell membranes and extract their contents. Combinations of such structures with piezoelectric elements for agitation can provide suitable shear forces for lysis. Such elements are described in greater detail with respect to nucleic acid fragmentation, below.

More traditional methods of cell extraction may also be used, e.g., employing a channel with restricted cross-sectional dimension which causes cell lysis when the sample is passed through the channel with sufficient flow pressure.

5 Alternatively, cell extraction and denaturing of contaminating proteins may be carried out by applying an alternating electrical current to the sample. More specifically, the sample of cells is flowed through a microtubular array while an alternating electric current is applied across the fluid

10 flow. A variety of other methods may be utilized within the device of the present invention to effect cell lysis/extraction, including, e.g., subjecting cells to ultrasonic agitation, or forcing cells through microgeometry apertures, thereby subjecting the cells to high shear stress

15 resulting in rupture.

Following extraction, it will often be desirable to separate the nucleic acids from other elements of the crude extract, e.g., denatured proteins, cell membrane particles, salts, and the like. Removal of particulate matter is generally accomplished by filtration, flocculation or the like. A variety of filter types may be readily incorporated into the device. Further, where chemical denaturing methods are used, it may be desirable to desalt the sample prior to proceeding to the next step. Desalting of the sample, and isolation of the nucleic acid may generally be carried out in a single step, e.g., by binding the nucleic acids to a solid phase and washing away the contaminating salts or performing gel filtration chromatography on the sample, passing salts through dialysis membranes, and the like. Suitable solid supports for nucleic acid binding include, e.g., diatomaceous earth, silica (i.e., glass wool), or the like. Suitable gel exclusion media, also well known in the art, may also be readily incorporated into the devices of the present invention, and is commercially available from, e.g., Pharmacia and Sigma Chemical.

The isolation and/or gel filtration/desalting may be carried out in an additional chamber, or alternatively, the

particular chromatographic media may be incorporated in a channel or fluid passage leading to a subsequent reaction chamber. Alternatively, the interior surfaces of one or more fluid passages or chambers may themselves be derivatized to 5 provide functional groups appropriate for the desired purification, e.g., charged groups, affinity binding groups and the like, i.e., poly-T oligonucleotides for mRNA purification.

Alternatively, desalting methods may generally take 10 advantage of the high electrophoretic mobility and negative of DNA compared to other elements. Electrophoretic methods may also be utilized in the purification of nucleic acids from other cell contaminants and debris. In one example, a separation channel or chamber of the device is fluidly 15 connected to two separate "field" channels or chambers having electrodes, e.g., platinum electrodes, disposed therein. The two field channels are separated from the separation channel using an appropriate barrier or "capture membrane" which allows for passage of current without allowing passage of 20 nucleic acids or other large molecules. The barrier generally serves two basic functions: first, the barrier acts to retain the nucleic acids which migrate toward the positive electrode within the separation chamber; and second, the barriers prevent the adverse effects associated with electrolysis at 25 the electrode from entering into the reaction chamber (e.g., acting as a salt junction). Such barriers may include, e.g., dialysis membranes, dense gels, PEI filters, or other suitable materials. Upon application of an appropriate electric field, the nucleic acids present in the sample will migrate toward 30 the positive electrode and become trapped on the capture membrane. Sample impurities remaining free of the membrane are then washed from the chamber by applying an appropriate fluid flow. Upon reversal of the voltage, the nucleic acids are released from the membrane in a substantially purer form. 35 The field channels may be disposed on the same or opposite sides or ends of a separation chamber or channel, and may be used in conjunction with mixing elements described herein, to

ensure maximal efficiency of operation. Further, coarse filters may also be overlaid on the barriers to avoid any fouling of the barriers by particulate matter, proteins or nucleic acids, thereby permitting repeated use.

5 In a similar aspect, the high electrophoretic mobility of nucleic acids with their negative charges, may be utilized to separate nucleic acids from contaminants by utilizing a short column of a gel or other appropriate matrix or gel which will slow or retard the flow of other 10 contaminants while allowing the faster nucleic acids to pass.

For a number of applications, it may be desirable to extract and separate messenger RNA from cells, cellular debris, and other contaminants. As such, the device of the present invention may, in some cases, include an mRNA 15 purification chamber or channel. In general, such purification takes advantage of the poly-A tails on mRNA. In particular and as noted above, poly-T oligonucleotides may be immobilized within a chamber or channel of the device to serve as affinity ligands for mRNA. Poly-T oligonucleotides may be 20 immobilized upon a solid support incorporated within the chamber or channel, or alternatively, may be immobilized upon the surface(s) of the chamber or channel itself. Immobilization of oligonucleotides on the surface of the 25 chambers or channels may be carried out by methods described herein including, e.g., oxidation and silanation of the surface followed by standard DMT synthesis of the oligonucleotides.

In operation, the lysed sample is introduced into this chamber or channel in a high salt solution to increase 30 the ionic strength for hybridization, whereupon the mRNA will hybridize to the immobilized poly-T. Hybridization may also be enhanced through incorporation of mixing elements, also as described herein. After enough time has elapsed for hybridization, the chamber or channel is washed with clean 35 salt solution. The mRNA bound to the immobilized poly-T oligonucleotides is then washed free in a low ionic strength buffer. The surface area upon which the poly-T

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oligonucleotides are immobilized may be increased through the use of etched structures within the chamber or channel, e.g., ridges, grooves or the like. Such structures also aid in the agitation of the contents of the chamber or channel, as 5 described herein. Alternatively, the poly-T oligonucleotides may be immobilized upon porous surfaces, e.g., porous silicon, zeolites silica xerogels, scintered particles, or other solid supports.

D. Amplification and In Vitro Transcription

10 Following sample collection and nucleic acid extraction, the nucleic acid portion of the sample is typically subjected to one or more preparative reactions. These preparative reactions include in vitro transcription, labeling, fragmentation, amplification and other reactions. 15 Nucleic acid amplification increases the number of copies of the target nucleic acid sequence of interest. A variety of amplification methods are suitable for use in the methods and device of the present invention, including for example, the polymerase chain reaction method or (PCR), the ligase chain 20 reaction (LCR), self sustained sequence replication (3SR), and nucleic acid based sequence amplification (NASBA).

The latter two amplification methods involve isothermal reactions based on isothermal transcription, which produce both single stranded RNA (ssRNA) and double stranded 25 DNA (dsDNA) as the amplification products in a ratio of approximately 30 or 100 to 1, respectively. As a result, where these latter methods are employed, sequence analysis may be carried out using either type of substrate, i.e., complementary to either DNA or RNA.

30 In particularly preferred aspects, the amplification step is carried out using PCR techniques that are well known in the art. See *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., Sninsky, J. and White, T., eds.) Academic Press (1990), incorporated herein by 35 reference in its entirety for all purposes. PCR amplification generally involves the use of one strand of the target nucleic acid sequence as a template for producing a large number of

complements to that sequence. Generally, two primer sequences complementary to different ends of a segment of the complementary strands of the target sequence hybridize with their respective strands of the target sequence, and in the presence of polymerase enzymes and nucleoside triphosphates, the primers are extended along the target sequence. The extensions are melted from the target sequence and the process is repeated, this time with the additional copies of the target sequence synthesized in the preceding steps. PCR amplification typically involves repeated cycles of denaturation, hybridization and extension reactions to produce sufficient amounts of the target nucleic acid. The first step of each cycle of the PCR involves the separation of the nucleic acid duplex formed by the primer extension. Once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target sequence. The primers are then extended to form complementary copies of the target strands. For successful PCR amplification, the primers are designed so that the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when separated from the template (complement), serves as a template for the extension of the other primer. The cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

In PCR methods, strand separation is normally achieved by heating the reaction to a sufficiently high temperature for a sufficient time to cause the denaturation of the duplex but not to cause an irreversible denaturation of the polymerase enzyme (see U.S. Patent No. 4,965,188, incorporated herein by reference). Typical heat denaturation involves temperatures ranging from about 80°C to 105°C for times ranging from seconds to minutes. Strand separation, however, can be accomplished by any suitable denaturing method including physical, chemical, or enzymatic means. Strand separation may be induced by a helicase, for example, or an

enzyme capable of exhibiting helicase activity. For example, the enzyme RecA has helicase activity in the presence of ATP. The reaction conditions suitable for strand separation by helicases are known in the art (see Kuhn Hoffman-Berling, 5 1978, *CSH-Quantitative Biology*, 43:63-67; and Radding, 1982, *Ann. Rev. Genetics* 16:405-436, each of which is incorporated herein by reference). Other embodiments may achieve strand separation by application of electric fields across the sample. For example, Published PCT Application Nos. WO 10 92/04470 and WO 95/25177, incorporated herein by reference, describe electrochemical methods of denaturing double stranded DNA by application of an electric field to a sample containing the DNA. Structures for carrying out this electrochemical denaturation include a working electrode, counter electrode 15 and reference electrode arranged in a potentiostat arrangement across a reaction chamber (See, Published PCT Application Nos. WO 92/04470 and WO 95/25177, each of which is incorporated herein by reference for all purposes). Such devices may be readily miniaturized for incorporation into the devices of the 20 present invention utilizing the microfabrication techniques described herein.

Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of at least 4 deoxyribonucleotide triphosphates 25 (typically selected from dATP, dGTP, dCTP, dUTP and dTTP) in a reaction medium which comprises the appropriate salts, metal cations, and pH buffering system. Reaction components and conditions are well known in the art (See *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., 30 Sninsky, J. and White, T., eds.) Academic Press (1990), previously incorporated by reference). Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis.

Published PCT Application No. WO 94/05414, to 35 Northrup and White, discusses the use of a microPCR chamber which incorporates microheaters and micropumps in the thermal cycling and mixing during the PCR reactions.

The amplification reaction chamber of the device may comprise a sealable opening for the addition of the various amplification reagents. However, in preferred aspects, the amplification chamber will have an effective amount of the 5 various amplification reagents described above, predisposed within the amplification chamber, or within an associated reagent chamber whereby the reagents can be readily transported to the amplification chamber upon initiation of the amplification operation. By "effective amount" is meant a 10 quantity and/or concentration of reagents required to carry out amplification of a targeted nucleic acid sequence. These amounts are readily determined from known PCR protocols. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual*, (2nd ed.) Vols. 1-3, Cold Spring Harbor Laboratory, (1989) and 15 *PCR Protocols: A Guide to Methods and Applications* (Innis, M., Gelfand, D., Sninsky, J. and White, T., eds.) Academic Press (1990), both of which are incorporated herein by reference for all purposes in their entirety. For those embodiments where the various reagents are predisposed within the amplification 20 or adjacent chamber, it will often be desirable for these reagents to be in lyophilized forms, to provide maximum shelf life of the overall device. Introduction of the liquid sample to the chamber then reconstitutes the reagents in active form, and the particular reactions may be carried out.

25 In some aspects, the polymerase enzyme may be present within the amplification chamber, coupled to a suitable solid support, or to the walls and surfaces of the amplification chamber. Suitable solid supports include those that are well known in the art, e.g., agarose, cellulose, 30 silica, divinylbenzene, polystyrene, etc. Coupling of enzymes to solid supports has been reported to impart stability to the enzyme in question, which allows for storage of days, weeks or even months without a substantial loss in enzyme activity, and without the necessity of lyophilizing the enzyme. The 94 kd, 35 single subunit DNA polymerase from *Thermus aquaticus* (or tag polymerase) is particularly suited for the PCR based amplification methods used in the present invention, and is

generally commercially available from, e.g., Promega, Inc., Madison, WI. In particular, monoclonal antibodies are available which bind the enzyme without affecting its polymerase activity. Consequently, covalent attachment of the 5 active polymerase enzyme to a solid support, or the walls of the amplification chamber can be carried out by using the antibody as a linker between the enzyme and the support.

In addition to PCR and IVT reactions, the methods and devices of the present invention are also applicable to a 10 number of other reaction types, e.g., reverse transcription, nick translation, and the like.

E. Labeling and Fragmentation

The nucleic acids in a sample will generally be labeled to facilitate detection in subsequent steps. Labeling 15 may be carried out during the amplification, *in vitro* transcription or nick translation processes. In particular, amplification, *in vitro* transcription or nick translation may incorporate a label into the amplified or transcribed sequence, either through the use of labeled primers or the 20 incorporation of labeled dNTPs into the amplified sequence.

Alternatively, the nucleic acids in the sample may be labeled following amplification. Post amplification labeling typically involves the covalent attachment of a particular detectable group upon the amplified sequences. 25 Suitable labels or detectable groups include a variety of fluorescent or radioactive labeling groups well known in the art. These labels may also be coupled to the sequences using methods that are well known in the art. See, e.g., Sambrook, et al.

30 In addition, amplified sequences may be subjected to other post amplification treatments. For example, in some cases, it may be desirable to fragment the sequence prior to hybridization with an oligonucleotide array, in order to provide segments which are more readily accessible to the 35 probes, which avoid looping and/or hybridization to multiple probes. Fragmentation of the nucleic acids may generally be carried out by physical, chemical or enzymatic methods that

are known in the art. These additional treatments may be performed within the amplification chamber, or alternatively, may be carried out in a separate chamber. For example, physical fragmentation methods may involve moving the sample 5 containing the nucleic acid over pits or spikes in the surface of a reaction chamber or fluid channel. The motion of the fluid sample, in combination with the surface irregularities produces a high shear rate, resulting in fragmentation of the nucleic acids. In one aspect, this may be accomplished in a 10 miniature device by placing a piezoelectric element, e.g., a PZT ceramic element adjacent to a substrate layer that covers a reaction chamber or flow channel, either directly, or through a liquid layer, as described herein. The substrate 15 layer has pits, spikes or apertures manufactured in the surface which are within the chamber or flow channel. By driving the PZT element in the thickness mode, a standing wave is set up within the chamber. Cavitation and/or streaming within the chamber results in substantial shear. Similar shear rates may be achieved by forcing the nucleic acid 20 containing fluid sample through restricted size flow passages, e.g., apertures having a cross-sectional dimension in the micron or submicron scale, thereby producing a high shear rate and fragmenting the nucleic acid.

A number of sample preparation operations may be 25 carried out by adjusting the pH of the sample, such as cell lysis, nucleic acid fragmentation, enzyme denaturation and the like. Similarly, pH control may also play a role in a wide variety of other reactions to be carried out in the device, i.e., for optimizing reaction conditions, neutralizing acid or 30 base additions, denaturing exogenously introduced enzymes, quenching reactions, and the like. Such pH monitoring and control may be readily accomplished using well known methods. For example, pH may be monitored by incorporation of a pH sensor or indicator within a particular chamber. Control may 35 then be carried out by titration of the chamber contents with an appropriate acid or base.

In an alternative aspect, the device may include an electronically controlled pH system. In operation, an electrode is placed adjacent, e.g., in fluid contact, to a reaction chamber while a counter electrode is positioned 5 within a second chamber or channel fluidly connected to the first. Upon application of current to these electrodes, the pH of the reaction chamber is altered through the electrolysis of water at the surface of the electrode, producing O₂ and hydrogen. A pH sensor may also be included within the 10 reaction chamber to provide for monitoring and/or feedback control of the precise pH within the chamber.

One example of a reaction chamber employing an electronic pH control system is shown in Figure 11. As shown, a device 1100 fabricated from two planar members 1102 and 15 1104, includes three distinct chambers, a reference chamber 1106, a reaction chamber 1108, and a counter-electrode chamber 1110. Each of the reference chamber 1106 and counter-electrode chamber 1110 are fluidly connected to the reaction chamber 1108, e.g., via fluid passages 1112 and 1114. These 20 passages are typically blocked by an appropriate barrier 1116, e.g., dialysis membrane, gel plug or the like, to prevent the electrophoretic passage of sample elements between the chambers. The reference chamber 1106 typically includes a reference electrode 1118. The reference electrode may be 25 fabricated, e.g., from a platinum, gold or nickel screen pressed with a mixture of teflon and platinum black (producing a hydrogen electrode). The reaction chamber 1108 typically includes an electrolysis electrode 1120, e.g., a platinum, gold or nickel screen coated with an appropriate barrier, 30 e.g., polyacrylamide gel layer, and a hydrogen electrode 1122, also protected with an appropriate barrier. The reference electrode 1118 and hydrogen electrode 1122 are connected to an electrometer 1126 for monitoring the pH within the reaction chamber. The counter-electrode chamber 1110 typically 35 includes the counter-electrode 1123, e.g., a single platinum, gold or nickel screen electrode. The electrolysis electrode

and counter-electrode are connected to an appropriate current source 1124.

Upon introduction of the sample, e.g., a cell suspension or nucleic acid containing sample, a current is applied by the current source. Electrolysis at the electrolysis electrode alters the pH within the reaction chamber 1108. The electrometer compares the pH sensed by the voltage between the reference and hydrogen electrodes. This signal may be compared to a set-point by appropriate means, e.g., an appropriately programmed computer or other microprocessor 1128, and used to control the application of current. The resulting system allows the automated control of pH within the reaction chamber by varying the set-point signal.

15 F. Sample Analysis

Following the various sample preparation operations, the sample will generally be subjected to one or more analysis operations. Particularly preferred analysis operations include, e.g., sequence based analyses using an oligonucleotide array and/or size based analyses using, e.g., microcapillary array electrophoresis.

1. Oligonucleotide Probe Array

In one aspect, following sample preparation, the nucleic acid sample is probed using an array of oligonucleotide probes. Oligonucleotide arrays generally include a substrate having a large number of positionally distinct oligonucleotide probes attached to the substrate. These oligonucleotide arrays, also described as "Genechip™ arrays," have been generally described in the art, for example, U.S. Patent No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092. These pioneering arrays may be produced using mechanical or light directed synthesis methods which incorporate a combination of photolithographic methods and solid phase oligonucleotide synthesis methods. See Fodor et al., *Science*, 251:767-777 (1991), Pirrung et al., U.S. Patent No. 5,143,854 (see also PCT Application No. WO 90/15070) and Fodor et al., PCT Publication No. WO 92/10092,

all incorporated herein by reference. These references disclose methods of forming vast arrays of peptides, oligonucleotides and other polymer sequences using, for example, light-directed synthesis techniques. Techniques 5 for the synthesis of these arrays using mechanical synthesis strategies are described in, e.g., PCT Publication No. 93/09668 and U.S. Patent No. 5,384,261, each of which is incorporated herein by reference in its entirety for all purposes. Incorporation of these arrays in injection molded 10 polymeric casings has been described in Published PCT Application No. 95/33846.

The basic strategy for light directed synthesis of oligonucleotide arrays is as follows. The surface of a solid support, modified with photosensitive protecting groups is 15 illuminated through a photolithographic mask, yielding reactive hydroxyl groups in the illuminated regions. A selected nucleotide, typically in the form of a 3'-O-phosphoramidite-activated deoxynucleoside (protected at the 5' hydroxyl with a photosensitive protecting group), is then 20 presented to the surface and coupling occurs at the sites that were exposed to light. Following capping and oxidation, the substrate is rinsed and the surface is illuminated through a second mask, to expose additional hydroxyl groups for coupling. A second selected nucleotide (e.g., 5'-protected, 25 3'-O-phosphoramidite-activated deoxynucleoside) is presented to the surface. The selective deprotection and coupling cycles are repeated until the desired set of products is obtained. Since photolithography is used, the process can be readily miniaturized to generate high density arrays of 30 oligonucleotide probes. Furthermore, the sequence of the oligonucleotides at each site is known. See, Pease, et al. Mechanical synthesis methods are similar to the light directed methods except involving mechanical direction of fluids for deprotection and addition in the synthesis steps.

35 Typically, the arrays used in the present invention will have a site density of greater than 100 different probes per cm^2 . Preferably, the arrays will have a site density of

greater than 500/cm², more preferably greater than about 1000/cm², and most preferably, greater than about 10,000/cm². Preferably, the arrays will have more than 100 different probes on a single substrate, more preferably greater than 5 about 1000 different probes still more preferably, greater than about 10,000 different probes and most preferably, greater than 100,000 different probes on a single substrate.

For some embodiments, oligonucleotide arrays may be prepared having all possible probes of a given length. Such 10 arrays may be used in such areas as sequencing or sequence checking applications, which offer substantial benefits over traditional methods. The use of oligonucleotide arrays in such applications is described in, e.g., U.S. Patent application Serial No. 08/515,919, filed July 24, 1995, and 15 U.S. Patent Application Serial No. 08/284,064, filed August 2, 1994, each of which is incorporated herein by reference in its entirety for all purposes. These methods typically use a set of short oligonucleotide probes of defined sequence to search for complementary sequences on a longer target strand of DNA. 20 The hybridization pattern of the target sequence on the array is used to reconstruct the target DNA sequence. Hybridization analysis of large numbers of probes can be used to sequence long stretches of DNA.

One strategy of *de novo* sequencing can be 25 illustrated by the following example. A 12-mer target DNA sequence is probed on an array having a complete set of octanucleotide probes. Five of the 65,536 octamer probes will perfectly hybridize to the target sequence. The identity of the probes at each site is known. Thus, by determining the 30 locations at which the target hybridizes on the array, or the hybridization pattern, one can determine the sequence of the target sequence. While these strategies have been proposed and utilized in some applications, there has been difficulty in demonstrating sequencing of larger nucleic acids using 35 these same strategies. Accordingly, in preferred aspects, SBH methods utilizing the devices described herein use data from mismatched probes, as well as perfectly matching probes, to

supply useful sequence data, as described in U.S. Patent Application No. 08/505,919, incorporated herein by reference.

While oligonucleotide probes may be prepared having every possible sequence of length n , it will often be 5 desirable in practicing the present invention to provide an oligonucleotide array which is specific and complementary to a particular nucleic acid sequence. For example, in particularly preferred aspects, the oligonucleotide array will contain oligonucleotide probes which are complementary to 10 specific target sequences, and individual or multiple mutations of these. Such arrays are particularly useful in the diagnosis of specific disorders which are characterized by the presence of a particular nucleic acid sequence. For example, the target sequence may be that of a particular 15 exogenous disease causing agent, e.g., human immunodeficiency virus (see, U.S. Application Serial No. 08/284,064, previously incorporated herein by reference), or alternatively, the target sequence may be that portion of the human genome which is known to be mutated in instances of a particular disorder, 20 i.e., sickle cell anemia (see, e.g., U.S. Application Serial No. 08/082,937, previously incorporated herein by reference) or cystic fibrosis.

In such an application, the array generally comprises at least four sets of oligonucleotide probes, 25 usually from about 9 to about 21 nucleotides in length. A first probe set has a probe corresponding to each nucleotide in the target sequence. A probe is related to its corresponding nucleotide by being exactly complementary to a subsequence of the target sequence that includes the 30 corresponding nucleotide. Thus, each probe has a position, designated an interrogation position, that is occupied by a complementary nucleotide to the corresponding nucleotide in the target sequence. The three additional probe sets each have a corresponding probe for each probe in the first probe 35 set, but substituting the interrogation position with the three other nucleotides. Thus, for each nucleotide in the target sequence, there are four corresponding probes, one from

each of the probe sets. The three corresponding probes in the three additional probe sets are identical to the corresponding probe from the first probe or a subsequence thereof that includes the interrogation position, except that the 5 interrogation position is occupied by a different nucleotide in each of the four corresponding probes.

Some arrays have fifth, sixth, seventh and eighth probe sets. The probes in each set are selected by analogous principles to those for the probes in the first four probe 10 sets, except that the probes in the fifth, sixth, seventh and eighth sets exhibit complementarity to a second reference sequence. In some arrays, the first set of probes is complementary to the coding strand of the target sequence while the second set is complementary to the noncoding strand. 15 Alternatively, the second reference sequence can be a subsequence of the first reference sequence having a substitution of at least one nucleotide.

In some applications, the target sequence has a substituted nucleotide relative to the probe sequence in at 20 least one undetermined position, and the relative specific binding of the probes indicates the location of the position and the nucleotide occupying the position in the target sequence.

Following amplification and/or labeling, the nucleic 25 acid sample is incubated with the oligonucleotide array in the hybridization chamber. Hybridization between the sample nucleic acid and the oligonucleotide probes upon the array is then detected, using, e.g., epifluorescence confocal microscopy. Typically, sample is mixed during hybridization 30 to enhance hybridization of nucleic acids in the sample to nucleic acid probes on the array. Again, mixing may be carried out by the methods described herein, e.g., through the use of piezoelectric elements, electrophoretic methods, or physical mixing by pumping fluids into and out of the 35 hybridization chamber, i.e., into an adjoining chamber. Generally, the detection operation will be performed using a reader device external to the diagnostic device. However, it

may be desirable in some cases, to incorporate the data gathering operation into the diagnostic device itself.

The hybridization data is next analyzed to determine the presence or absence of a particular sequence within the 5 sample, or by analyzing multiple hybridizations to determine the sequence of the target nucleic acid using the SBH techniques already described.

In some cases, hybridized oligonucleotides may be labeled following hybridization. For example, where biotin 10 labeled dNTPs are used in, e.g., amplification or transcription, streptavidin linked reporter groups may be used to label hybridized complexes. Such operations are readily integratable into the systems of the present invention.

2. Capillary Electrophoresis

15 In some embodiments, it may be desirable to provide an additional, or alternative means for analyzing the nucleic acids from the sample. In one embodiment, the device of the invention will optionally or additionally comprise a micro capillary array for analysis of the nucleic acids obtained 20 from the sample.

Microcapillary array electrophoresis generally involves the use of a thin capillary or channel which may or "may not be filled with a particular separation medium.

Electrophoresis of a sample through the capillary provides a 25 size based separation profile for the sample. The use of microcapillary electrophoresis in size separation of nucleic acids has been reported in, e.g., Woolley and Mathies, *Proc. Nat'l Acad. Sci. USA* (1994) 91:11348-11352. Microcapillary array electrophoresis generally provides a rapid method for 30 size based sequencing, PCR product analysis and restriction fragment sizing. The high surface to volume ratio of these capillaries allows for the application of higher electric fields across the capillary without substantial thermal variation across the capillary, consequently allowing for more 35 rapid separations. Furthermore, when combined with confocal imaging methods, these methods provide sensitivity in the

range of attomoles, which is comparable to the sensitivity of radioactive sequencing methods.

Microfabrication of microfluidic devices including microcapillary electrophoretic devices has been discussed in detail in, e.g., Jacobsen, et al., *Anal. Chem.* (1994) 66:1114-1118, Effenhauser, et al., *Anal. Chem.* (1994) 66:2949-2953, Harrison, et al., *Science* (1993) 261:895-897, Effenhauser, et al. *Anal. Chem.* (1993) 65:2637-2642, and Manz, et al., *J. Chromatog.* (1992) 593:253-258. Typically, these methods comprise photolithographic etching of micron scale channels on a silica, silicon or other rigid substrate or chip, and can be readily adapted for use in the miniaturized devices of the present invention. In some embodiments, the capillary arrays may be fabricated from the same polymeric materials described for the fabrication of the body of the device, using the injection molding techniques described herein. In such cases, the capillary and other fluid channels may be molded into a first planar element. A second thin polymeric member having ports corresponding to the termini of the capillary channels disposed therethrough, is laminated or sonically welded onto the first to provide the top surface of these channels. Electrodes for electrophoretic control are disposed within these ports/wells for application of the electrical current to the capillary channels. Through use of a relatively thin sheet as the covering member of the capillary channels, heat generated during electrophoresis can be rapidly dissipated. Additionally, the capillary channels may be coated with more thermally conductive material, e.g., glass or ceramic, to enhance heat dissipation.

In many capillary electrophoresis methods, the capillaries, e.g., fused silica capillaries or channels etched, machined or molded into planar substrates, are filled with an appropriate separation/sieving matrix. Typically, a variety of sieving matrices are known in the art may be used in the microcapillary arrays. Examples of such matrices include, e.g., hydroxyethyl cellulose, polyacrylamide, agarose and the like. Gel matrices may be introduced and polymerized

within the capillary channel. However, in some cases, this may result in entrapment of bubbles within the channels which can interfere with sample separations. Accordingly, it is often desirable to place a preformed separation matrix within 5 the capillary channel(s), prior to mating the planar elements of the capillary portion. Fixing the two parts, e.g., through sonic welding, permanently fixes the matrix within the channel. Polymerization outside of the channels helps to ensure that no bubbles are formed. Further, the pressure of 10 the welding process helps to ensure a void-free system. Generally, the specific gel matrix, running buffers and running conditions are selected to maximize the separation characteristics of the particular application, e.g., the size of the nucleic acid fragments, the required resolution, and 15 the presence of native or undenatured nucleic acid molecules. For example, running buffers may include denaturants, chaotropic agents such as urea or the like, to denature nucleic acids in the sample.

In addition to its use in nucleic acid 20 "fingerprinting" and other sized based analyses, the capillary arrays may also be used in sequencing applications. In particular, gel based sequencing techniques may be readily adapted for capillary array electrophoresis. For example, capillary electrophoresis may be combined with the Sanger 25 dideoxy chain termination sequencing methods as discussed in Sambrook, et al. (See also Brenner, et al., *Proc. Nat'l Acad. Sci.* (1989) 86:8902-8906). In these methods, the sample nucleic acid is amplified in the presence of fluorescent 30 dideoxynucleoside triphosphates in an extension reaction. The random incorporation of the dideoxynucleotides terminates transcription of the nucleic acid. This results in a range of transcription products differing from another member by a single base. Comparative size based separation then allows 35 the sequence of the nucleic acid to be determined based upon the last dideoxy nucleotide to be incorporated.

G. Data Gathering and Analysis

Gathering data from the various analysis operations, e.g., oligonucleotide and/or microcapillary arrays, will typically be carried out using methods known in the art. For 5 example, the arrays may be scanned using lasers to excite fluorescently labeled targets that have hybridized to regions of probe arrays, which can then be imaged using charged coupled devices ("CCDs") for a wide field scanning of the array. Alternatively, another particularly useful method for 10 gathering data from the arrays is through the use of laser confocal microscopy which combines the ease and speed of a readily automated process with high resolution detection. Particularly preferred scanning devices are generally described in, e.g., U.S. Patent Nos. 5,143,854 and 5,424,186.

15 Following the data gathering operation, the data will typically be reported to a data analysis operation. To facilitate the sample analysis operation, the data obtained by the reader from the device will typically be analyzed using a digital computer. Typically, the computer will be 20 appropriately programmed for receipt and storage of the data from the device, as well as for analysis and reporting of the data gathered, i.e., interpreting fluorescence data to determine the sequence of hybridizing probes, normalization of background and single base mismatch hybridizations, ordering 25 of sequence data in SBH applications, and the like, as described in, e.g., U.S. Patent Application Serial No. 08/327,525, filed October 21, 1994, and incorporated herein by reference.

30 III. The Nucleic Acid Diagnostic System

A. Analytical System

A schematic of a representative analytical system based upon the device of the invention is shown in Figure 1. The system includes the diagnostic device 2 which performs one 35 or more of the operations of sample collection, preparation and/or analysis using, e.g., hybridization and/or size based separation. The diagnostic device is then placed in a reader

device 4 to detect the hybridization and or separation information present on the device. The hybridization and/or separation data is then reported from the reader device to a computer 6 which is programmed with appropriate software for 5 interpreting the data obtained by the reader device from the diagnostic device. Interpretation of the data from the diagnostic device may be used in a variety of ways, including nucleic acid sequencing which is directed toward a particular disease causing agent, such as viral or bacterial infections, 10 e.g., AIDS, malaria, etc., or genetic disorders, e.g., sickle cell anemia, cystic fibrosis, Fragile X syndrome, Duchenne muscular dystrophy, and the like. Alternatively, the device can be employed in de novo sequencing applications to identify the nucleic acid sequence of a previously unknown sequence.

15 B. The Diagnostic Device

1. Generally

As described above, the device of the present invention is generally capable of carrying out a number of preparative and analytical reactions on a sample. To achieve 20 this end, the device generally comprises a number of discrete reaction, storage and/or analytical chambers disposed within a single unit or body. While referred to herein as a "diagnostic device," those of skill in the art will appreciate that the device of the invention will have a variety of 25 applications outside the scope of diagnostics, alone. Such applications include sequencing applications, sample identification and characterization applications (for, e.g., taxonomic studies, forensic applications, i.e., criminal investigations, and the like).

30 Typically, the body of the device defines the various reaction chambers and fluid passages in which the above described operations are carried out. Fabrication of the body, and thus the various chambers and channels disposed within the body may generally be carried out using one or a 35 combination of a variety of well known manufacturing techniques and materials. Generally, the material from which the body is fabricated will be selected so as to provide

maximum resistance to the full range of conditions to which the device will be exposed, e.g., extremes of temperature, salt, pH, application of electric fields and the like, and will also be selected for compatibility with other materials used in the device. Additional components may be later introduced, as necessary, into the body. Alternatively, the device may be formed from a plurality of distinct parts that are later assembled or mated. For example, separate and individual chambers and fluid passages may be assembled to provide the various chambers of the device.

As a miniaturized device, the body of the device will typically be approximately 1 to 20 cm in length by about 1 to 10 cm in width by about 0.1 to about 2 cm thick.

Although indicative of a rectangular shape, it will be readily appreciated that the devices of the invention may be embodied in any number of shapes depending upon the particular need. Additionally, these dimensions will typically vary depending upon the number of operations to be performed by the device, the complexity of these operations and the like. As a result, these dimensions are provided as a general indication of the size of the device. The number and size of the reaction chambers included within the device will also vary depending upon the specific application for which the device is to be used. Generally, the device will include at least two distinct reaction chambers, and preferably, at least three, four or five distinct reaction chambers, all integrated within a single body. Individual reaction chambers will also vary in size and shape according to the specific function of the reaction chamber. For example, in some cases, circular reaction chambers may be employed. Alternatively, elongate reaction chambers may be used. In general however, the reaction chambers will be from about 0.05 to about 20 mm in width or diameter, preferably from about 0.1 or 0.5 to about 20 mm in width or diameter and about 0.05 to about 5 mm deep, and preferably 0.05 to about 1 mm deep. For elongate chambers, length will also typically vary along these same ranges. Fluid channels, on the other hand, are typically

distinguished from chambers in having smaller dimensions relative to the chambers, and will typically range from about 10 to about 1000 μm wide, preferably, 100 to 500 μm wide and about 1 to 500 μm deep. Although described in terms of 5 reaction chambers, it will be appreciated that these chambers may perform a number of varied functions, e.g., as storage chambers, incubation chambers, mixing chambers and the like.

In some cases, a separate chamber or chambers may be used as volumetric chambers, e.g., to precisely measure fluid 10 volumes for introduction into a subsequent reaction chamber. In such cases, the volume of the chamber will be dictated by volumetric needs of a given reaction. Further, the device may be fabricated to include a range of volumetric chambers having varied, but known volumes or volume ratios (e.g., in 15 comparison to a reaction chamber or other volumetric chambers).

As described above, the body of the device is generally fabricated using one or more of a variety of methods and materials suitable for microfabrication techniques. For 20 example, in preferred aspects, the body of the device may comprise a number of planar members that may individually be injection molded parts fabricated from a variety of polymeric materials, or may be silicon, glass, or the like. In the case of substrates like silica, glass or silicon, methods for 25 etching, milling, drilling, etc., may be used to produce wells and depressions which make up the various reaction chambers and fluid channels within the device. Microfabrication techniques, such as those regularly used in the semiconductor and microelectronics industries are particularly suited to 30 these materials and methods. These techniques include, e.g., electrodeposition, low-pressure vapor deposition, photolithography, wet chemical etching, reactive ion etching (RIE), laser drilling, and the like. Where these methods are used, it will generally be desirable to fabricate the planar 35 members of the device from materials similar to those used in the semiconductor industry, i.e., silica, silicon, gallium arsenide, polyimide substrates. U.S. Patent No. 5,252,294, to

Kroy, et al., incorporated herein by reference in its entirety for all purposes, reports the fabrication of a silicon based multiwell apparatus for sample handling in biotechnology applications.

5 Photolithographic methods of etching substrates are particularly well suited for the microfabrication of these substrates and are well known in the art. For example, the first sheet of a substrate may be overlaid with a photoresist. An electromagnetic radiation source may then be shone through
10 a photolithographic mask to expose the photoresist in a pattern which reflects the pattern of chambers and/or channels on the surface of the sheet. After removing the exposed photoresist, the exposed substrate may be etched to produce the desired wells and channels. Generally preferred
15 photoresists include those used extensively in the semiconductor industry. Such materials include polymethyl methacrylate (PMMA) and its derivatives, and electron beam resists such as poly(olefin sulfones) and the like (more fully discussed in, e.g., Ghandi, "VLSI Fabrication Principles,"
20 Wiley (1983) Chapter 10, incorporated herein by reference in its entirety for all purposes).

As an example, the wells manufactured into the surface of one planar member make up the various reaction chambers of the device. Channels manufactured into the
25 surface of this or another planar member make up fluid channels which are used to fluidly connect the various reaction chambers. Another planar member is then placed over and bonded to the first, whereby the wells in the first planar member define cavities within the body of the device which
30 cavities are the various reaction chambers of the device. Similarly, fluid channels manufactured in the surface of one planar member, when covered with a second planar member define fluid passages through the body of the device. These planar members are bonded together or laminated to produce a fluid
35 tight body of the device. Bonding of the planar members of the device may generally be carried out using a variety of methods known in the art and which may vary depending upon the

materials used. For example, adhesives may generally be used to bond the planar members together. Where the planar members are, e.g., glass, silicon or combinations thereof, thermal bonding, anodic/electrostatic or silicon fusion bonding 5 methods may be applied. For polymeric parts, a similar variety of methods may be employed in coupling substrate parts together, e.g., heat with pressure, solvent based bonding. Generally, acoustic welding techniques are generally preferred. In a related aspect, this adhesive tapes may be 10 employed as one portion of the device forming a thin wall of the reaction chamber/channel structures.

Although primarily described in terms of producing a fully integrated body of the device, the above described methods can also be used to fabricate individual discrete 15 components of the device which are later assembled into the body of the device.

In additional embodiments, the body may comprise a combination of materials and manufacturing techniques described above. In some cases, the body may include some 20 parts of injection molded plastics, and the like, while other portions of the body may comprise etched silica or silicon planar members, and the like. For example, injection molding techniques may be used to form a number of discrete cavities in a planar surface which define the various reaction 25 chambers, whereas additional components, e.g., fluid channels, arrays, etc, may be fabricated on a planar glass, silica or silicon chip or substrate. Lamination of one set of parts to the other will then result in the formation of the various reaction chambers, interconnected by the appropriate fluid 30 channels.

In particularly preferred embodiments, the body of the device is made from at least one injection molded, press molded or machined polymeric part that has one or more wells or depressions manufactured into its surface to define several 35 of the walls of the reaction chamber or chambers. Molds or mold faces for producing these injection molded parts may generally be fabricated using the methods described herein

for, e.g., silicon molds. Examples of suitable polymers for injection molding or machining include, e.g., polycarbonate, polystyrene, polypropylene, polyethylene, acrylic, and commercial polymers such as Kapton, Valox, Teflon, ABS, Delrin and the like. A second part that is similarly planar in shape is mated to the surface of the polymeric part to define the remaining wall of the reaction chamber(s). Published PCT Application No. 95/33846, incorporated herein by reference, describes a device that is used to package individual oligonucleotide arrays. The device includes a hybridization chamber disposed within a planar body. The chamber is fluidly connected to an inlet port and an outlet port via flow channels in the body of the device. The body includes a plurality of injection molded planar parts that are mated to form the body of the device, and which define the flow channels and hybridization chamber.

The surfaces of the fluid channels and reaction chambers which contact the samples and reagents may also be modified to better accomodate a desired reaction. Surfaces may be made more hydrophobic or more hydrophilic depending upon the particular application. Alternatively, surfaces may be coated with any number of materials in order to make the overall system more compatible to the reactions being carried out. For example, in the case of nucleic acid analyses, it may be desireable to coat the surfaces with, e.g., a teflon or other non-stick coating, to prevent adhesion of nucleic acids to the surface. Additionally, insulator coatings may also be desirable in those instances where electrical leads are placed in contact with fluids, to prevent shorting out, or excess gas formation from electrolysis. Such insulators may include those well known in the art, e.g., silicon oxide, ceramics or the like. Additional surface treatments are described in greater detail below.

Figures 2A and 2B show a schematic representation of one embodiment of a reaction chamber for inclusion in the device of the invention. The reaction chamber includes a machined or injection molded polymeric part 102 which has a

well 104 manufactured, i.e., machined or molded, into its surface. This well may be closed at the end opposite the well opening as shown in Figure 2A, or optionally, may be supplied with an additional opening 118 for inclusion of an optional 5 vent, as shown in Figure 2B.

The reaction chamber is also provided with additional elements for transporting a fluid sample to and from the reaction chamber. These elements include one or more fluid channels (122 and 110 in Figures 2A and 2B, 10 respectively) which connect the reaction chamber to an inlet/outlet port for the overall device, additional reaction chambers, storage chambers or one or more analytical chambers.

A second part 124, typically planar in structure, is mated to the polymeric part to define a closure for the 15 reaction chamber. This second part may incorporate the fluid channels, as shown in Figures 2A and 2B, or may merely define a further wall of the fluid channels provided in the surface of the first polymeric part (not shown). Typically, this second part will comprise a series of fluid channels 20 manufactured into one of its surfaces, for fluidly connecting the reaction chamber to an inlet port in the overall device or to another reaction or analytical chamber. Again, this second part may be a second polymeric part made by injection molding or machining techniques. Alternatively, this second part may 25 be manufactured from a variety of other materials, including glass, silica, silicon or other crystalline substrates. Microfabrication techniques suited for these substrates are generally well known in the art and are described above.

In a first preferred embodiment, the reaction 30 chamber is provided without an inlet/outlet valve structure, as shown in Figure 2A. For these embodiments, the fluid channels 122 may be provided in the surface of the second part that is mated with the surface of the polymeric part such that upon mating the second part to the first polymeric part, the 35 fluid channel 122 is fluidly connected to the reaction chamber 104.

Alternatively, in a second preferred embodiment, the reaction chamber may be provided with an inlet/outlet valve structure for sealing the reaction chamber to retain a fluid sample therein. An example of such a valve structure is shown 5 in Figure 2B. In particular, the second part 124 mated to the polymeric part may comprise a plurality of mated planar members, wherein a first planar member 106 is mated with the first polymeric part 102 to define a wall of the reaction chamber. The first planar member 106 has an opening 108 disposed therethrough, defining an inlet to the reaction 10 chamber. This first planar member also includes a fluid channel 110 etched in the surface opposite the surface that is mated with the first polymeric part 102. The fluid channel terminates adjacent to, but not within the reaction chamber 15 inlet 108. The first planar member will generally be manufactured from any of the above described materials, using the above-described methods. A second planar member 112 is mated to the first and includes a diaphragm valve 114 which extends across the inlet 108 and overlaps with the fluid 20 channel 110 such that deflection of the diaphragm results in a gap between the first and second planar members, thereby creating a fluid connection between the reaction chamber 104 and the fluid channel 110, via the inlet 108. Deflection of the diaphragm valve may be carried out by a variety of methods 25 including, e.g., application of a vacuum, electromagnetic and/or piezoelectric actuators coupled to the diaphragm valve, and the like. To allow for a deflectable diaphragm, the second planar member will typically be fabricated, at least in part, from a flexible material, e.g., silicon, silicone, 30 latex, mylar, polyimide, Teflon or other flexible polymers. As with the reaction chambers and fluid channels, these diaphragms will also be of miniature scale. Specifically, valve and pump diaphragms used in the device will typically range in size depending upon the size of the chamber or fluid 35 passage to which they are fluidly connected. In general, however, these diaphragms will be in the range of from about 0.5 to about 5 mm for valve diaphragms, and from about 1 to

about 20 mm in diameter for pumping diaphragms. As shown in Figure 2B, second part 124 includes an additional planar member 116 having an opening 126 for application of pressure or vacuum for deflection of valve 114.

5 Where reagents involved in a particular analysis are incompatible with the materials used to manufacture the device, e.g., silicon, glass or polymeric parts, a variety of coatings may be applied to the surfaces of these parts that contact these reagents. For example, components that have
10 silicon elements may be coated with a silicon nitride layer or a metallic layer of, e.g., gold or nickel, may be sputtered or electroplated on the surface to avoid adverse reactions with these reagents. Similarly, inert polymer coatings, e.g., Teflon and the like, pyraline coatings, or surface silanation
15 modifications may also be applied to internal surfaces of the chambers and/or channels.

20 The reaction/storage chamber 104 shown in Figure 2B is also shown with an optional vent 118, for release of displaced gas present in the chamber when the fluid is introduced. In preferred aspects, this vent may be fitted with a gas permeable fluid barrier 120, which permits the passage of gas without allowing for the passage of fluid, e.g., a poorly wetting filter plug. A variety of materials are suitable for use as poorly wetting filter plugs including,
25 e.g., porous hydrophobic polymer materials, such as spun fibers of acrylic, polycarbonate, teflon, pressed polypropylene fibers, or any number commercially available filter plugs (American Filtrona Corp., Richmond, VA, Gelman Sciences, and the like). Alternatively, a hydrophobic membrane can be bonded over a thru-hole to supply a similar structure. Modified acrylic copolymer membranes are commercially available from, e.g., Gelman Sciences (Ann Arbor, MI) and particle-track etched polycarbonate membranes are available from Poretics, Inc. (Livermore, CA). Venting of
30 heated chambers may incorporate barriers to evaporation of the sample, e.g., a reflux chamber or a mineral oil layer disposed within the chamber, and over the top surface of the sample, to
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permit the evolution of gas while preventing excessive evaporation of fluid from the sample.

As described herein, the overall geometry of the device of the invention may take a number of forms. For example, the device may incorporate a plurality of reaction chambers, storage chambers and analytical chambers, arranged in series, whereby a fluid sample is moved serially through the chambers, and the respective operations performed in these chambers. Alternatively, the device may incorporate a central fluid distribution channel or chamber having the various reaction/storage/analytical chambers arranged around and fluidly connected to the central channel or chamber, which central channel or chamber acts as a conduit or hub for sample redistribution to the various chambers.

An example of the serial geometry of the device is shown in Figure 3. In particular, the illustrated device includes a plurality of reaction/storage/analytical chambers for performing a number of the operations described above, fluidly connected in series.

The schematic representation of the device in Figure 3 shows a device that comprises several reaction chambers arranged in a serial geometry. Specifically, the body of the device 200 incorporates reaction chambers 202, 206, 210, 214 and 218. These chambers are fluidly connected in series by fluid channels 208, 212 and 216, respectively.

In carrying out the various operations outlined above, each of these reaction chambers is assigned one or more different functions. For example, reaction chamber 202 may be a sample collection chamber which is adapted for receiving a fluid sample, i.e., a cell containing sample. For example, this chamber may include an opening to the outside of the device adapted for receipt of the sample. The opening will typically incorporate a sealable closure to prevent leakage of the sample, e.g., a valve, check-valve, or septum, through which the sample is introduced or injected. In some embodiments, the apparatus may include a hypodermic needle or other sample conduit, integrated into the body of the device

and in fluid connection with the sample collection chamber, for direct transfer of the sample from the host, patient, sample vial or tube, or other origin of the sample to the sample collection chamber.

5 Additionally, the sample collection chamber may have disposed therein, a reagent or reagents for the stabilization of the sample for prolonged storage, as described above. Alternatively, these reagents may be disposed within a reagent storage chamber adjacent to and fluidly connected with the
10 sample collection chamber.

15 The sample collection chamber is connected via a first fluid channel 204 to second reaction chamber 210 in which the extraction of nucleic acids from the cells within the sample may be performed. This is particularly suited to analytical operations to be performed where the samples include whole cells. The extraction chamber will typically be connected to sample collection chamber, however, in some cases, the extraction chamber may be integrated within and exist as a portion of the sample collection chamber. As
20 previously described, the extraction chamber may include physical and or chemical means for extracting nucleic acids from cells.

25 The extraction chamber is fluidly connected via a second fluid channel 208, to third reaction chamber 210 in which amplification of the nucleic acids extracted from the sample is carried out. The amplification process begins when the sample is introduced into the amplification chamber. As described previously, amplification reagents may be exogenously introduced, or will preferably be predisposed
30 within the reaction chamber. However, in alternate embodiments, these reagents will be introduced to the amplification chamber from an optional adjacent reagent chamber or from an external source through a sealable opening in the amplification chamber.

35 For PCR amplification methods, denaturation and hybridization cycling will preferably be carried out by repeated heating and cooling of the sample. Accordingly, PCR

based amplification chambers will typically include a a
temperature controller for heating the reaction to carry out
the thermal cycling. For example, a heating element or
temperature control block may be disposed adjacent the
5 external surface of the amplification chamber thereby
transferring heat to the amplification chamber. In this case,
preferred devices will include a thin external wall for
chambers in which thermal control is desired. This thin wall
may be a thin cover element, e.g., polycarbonate sheet, or
10 high temperature tape, i.e. silicone adhesive on Kapton tape
(commercially available from, e.g., 3M Corp.). Micro-scale
PCR devices have been previously reported. For example,
published PCT Application No. WO 94/05414, to Northrup and
White reports a miniaturized reaction chamber for use as a PCR
15 chamber, incorporating microheaters, e.g., resistive heaters.
The high surface area to volume ratio of the chamber allows
for very rapid heating and cooling of the reagents disposed
therein. Similarly, U.S. Patent No. 5,304,487 to Wilding et
al., previously incorporated by reference, also discusses the
20 use of a microfabricated PCR device.

In preferred embodiments, the amplification chamber
will incorporate a controllable heater disposed within or
adjacent to the amplification chamber, for thermal cycling of
the sample. Thermal cycling is carried out by varying the
25 current supplied to the heater to achieve the desired
temperature for the particular stage of the reaction.
Alternatively, thermal cycling for the PCR reaction may be
achieved by transferring the fluid sample among a number of
different reaction chambers or regions of the same reaction
30 chamber, having different, although constant temperatures, or
by flowing the sample through a serpentine channel which
travels through a number of varied temperature 'zones'.
Heating may alternatively be supplied by exposing the
amplification chamber to a laser or other light or
35 electromagnetic radiation source.

The amplification chamber is fluidly connected via a
fluid channel, e.g., fluid channel 212, to an additional

reaction chamber 214 which can carry out additional preparative operations, such as labeling or fragmentation.

A fourth fluid channel 216 connects the labeling or fragmentation chamber to an analytical chamber 218. As shown, 5 the analytical chamber includes an oligonucleotide array 220 as the bottom surface of the chamber. Analytical chamber 218 may optionally, or additionally comprise a microcapillary electrophoresis device 226 and additional preparative reaction chambers, e.g., 224 for performing, e.g., extension reactions, 10 fluidly connected to, e.g., chamber 210. The analytical chamber will typically have as at least one surface, a transparent window for observation or scanning of the particular analysis being performed.

Figures 4A-C illustrate an embodiment of a 15 microcapillary electrophoresis device. In this embodiment, the sample to be analyzed is introduced into sample reservoir 402. This sample reservoir may be a separate chamber, or may be merely a portion of the fluid channel leading from a previous reaction chamber. Reservoirs 404, 406 and 414 are 20 filled with sample/running buffer. Figure 4A illustrates the loading of the sample by plug loading, where the sample is drawn across the intersection of loading channel 416 and capillary channel 412, by application of an electrical current across buffer reservoir 406 and sample reservoir 402. In 25 alternative embodiments, the sample is "stack" loaded by applying an electrical current across sample reservoir 402 and waste reservoir 414, as shown in Figure 4B. Following sample loading, an electrical field is applied across buffer reservoir 404 and waste reservoir 414, electrophoresing the 30 sample through the capillary channel 412. Running of the sample is shown in Figure 4C. Although only a single capillary is shown in Figures 4A-C, the device of the present invention may typically comprise more than one capillary, and more typically, will comprise an array of four or more 35 capillaries, which are run in parallel. Fabrication of the microcapillary electrophoresis device may generally be carried using the methods described herein and as described in e.g.,

Woolley and Mathies, Proc. Nat'l Acad. Sci. USA 91:11348-11352 (1994), incorporated herein by reference in its entirety for all purposes. Typically, each capillary will be fluidly connected to a separate extension reaction chamber for 5 incorporation of a different dideoxynucleotide.

An alternate layout of the reaction chambers within the device of the invention, as noted above, includes a centralized geometry having a central chamber for gathering and distribution of a fluid sample to a number of separate 10 reaction/storage/analytical chambers arranged around, and fluidly connected to the central chamber. An example of this centralized geometry is shown in Figure 5. In the particular device shown, a fluid sample is introduced into the device through sample inlet 502, which is typically fluidly connected 15 to a sample collection chamber 504. The fluid sample is then transported to a central chamber 508 via fluid channel 506. Once within the central chamber, the sample may be transported to any one of a number of reaction/storage/analytical chambers (510, 512, 514) which are arranged around and fluidly 20 connected to the central chamber. As shown, each of reaction chambers 510, 512 and 514, includes a diaphragm 516, 518 and 520, respectively, as shown in Figure 2B, for opening and closing the fluid connection between the central chamber 508 and the reaction chamber. Additional reaction chambers may be 25 added fluidly connected to the central chamber, or alternatively, may be connected to any of the above described reaction chambers.

In certain aspects, the central chamber may have a dual function as both a hub and a pumping chamber. In 30 particular, this central pumping chamber can be fluidly connected to one or more additional reaction and/or storage chambers and one or more analytical chambers. The central pumping chamber again functions as a hub for the various operations to be carried out by the device as a whole as 35 described above. This embodiment provides the advantage of a single pumping chamber to deliver a sample to numerous operations, as well as the ability to readily incorporate

additional sample preparation operations within the device by opening another valve on the central pumping chamber.

In particular, the central chamber 508 may incorporate a diaphragm pump as one surface of the chamber, and in preferred aspects, will have a zero displacement when the diaphragm is not deflected. The diaphragm pump will generally be similar to the valve structure described above for the reaction chamber. For example, the diaphragm pump will generally be fabricated from any one of a variety of flexible materials, e.g., silicon, latex, teflon, mylar and the like. In particularly preferred embodiments, the diaphragm pump is silicon.

With reference to both Figures 5A and 5B, central chamber 508 is fluidly connected to sample collection chamber 504, via fluid channel 506. The sample collection chamber end of fluid channel 506 includes a diaphragm valve 524 for arresting fluid flow. A fluid sample is typically introduced into sample collection chamber through a sealable opening 502 in the body of the device, e.g., a valve or septum. Additionally, sample chamber 504 may incorporate a vent to allow displacement of gas or fluid during sample introduction.

Once the sample is introduced into the sample collection chamber, it may be drawn into the central pumping chamber 508 by the operation of pump diaphragm 526. Specifically, opening of sample chamber valve 524 opens fluid channel 506. Subsequent pulling or deflection of pump diaphragm 526 creates negative pressure within pumping chamber 508, thereby drawing the sample through fluid channel 506 into the central chamber. Subsequent closing of the sample chamber valve 524 and relaxation of pump diaphragm 526, creates a positive pressure within pumping chamber 508, which may be used to deliver the sample to additional chambers in the device. For example, where it is desired to add specific reagents to the sample, these reagents may be stored in liquid or solid form within an adjacent storage chamber 510. Opening valve 516 opens fluid channel 528, allowing delivery of the sample into storage chamber 510 upon relaxation of the

diaphragm pump. The operation of pumping chamber may further be employed to mix reagents, by repeatedly pulling and pushing the sample/reagent mixture to and from the storage chamber. This has the additional advantage of eliminating the necessity 5 of including additional mixing components within the device. Additional chamber/valve/fluid channel structures may be provided fluidly connected to pumping chamber 508 as needed to provide reagent storage chambers, additional reaction chambers or additional analytical chambers. Figure 5A illustrates an 10 additional reaction/storage chamber 514 and valve 520, fluidly connected to pumping chamber 508 via fluid channel 530. This will typically vary depending upon the nature of the sample to be analyzed, the analysis to be performed, and the desired sample preparation operation. Following any sample 15 preparation operation, opening valve 520 and closure of other valves to the pumping chamber, allows delivery of the sample through fluid channels 530 and 532 to reaction chamber 514, which may include an analytical device such as an oligonucleotide array for determining the hybridization of 20 nucleic acids in the sample to the array, or a microcapillary electrophoresis device for performing a size based analysis of the sample.

The transportation of fluid within the device of the invention may be carried out by a number of varied methods. 25 For example, fluid transport may be affected by the application of pressure differentials provided by either external or internal sources. Alternatively, internal pump elements which are incorporated into the device may be used to transport fluid samples through the device.

30 In a first embodiment, fluid samples are moved from one reaction/storage/analytical chamber to another chamber via fluid channels by applying a positive pressure differential from the originating chamber, the chamber from which the sample is to be transported, to the receiving chamber, the 35 chamber to which the fluid sample is to be transported. In order to apply the pressure differentials, the various reaction chambers of the device will typically incorporate

pressure inlets connecting the reaction chamber to the pressure source (positive or negative). For ease of discussion, the application of a negative pressure, i.e., to the receiving chamber, will generally be described herein.

5 However, upon reading the instant disclosure, one of ordinary skill in the art will appreciate that application of positive pressure, i.e., to the originating chamber, will be as effective, with only slight modifications, which will be illustrated as they arise herein.

10 In one method, application of the pressure differential to a particular reaction chamber may generally be carried out by selectively lowering the pressure in the receiving chamber. Selective lowering of the pressure in a particular receiving chamber may be carried out by a variety 15 of methods. For example, the pressure inlet for the reaction chambers may be equipped with a controllable valve structure which may be selectively operated to be opened to the pressure source. Application of the pressure source to the sample chamber then forces the sample into the next reaction chamber 20 which is at a lower pressure.

Typically, the device will include a pressure/vacuum manifold for directing an external vacuum source to the various reaction/storage/analytical chambers. A particularly elegant example of a preferred vacuum pressure manifold is 25 illustrated in Figures 6A, 6B and 6C.

The vacuum/pressure manifold produces a stepped pressure differential between each pair of connected reaction chambers. For example, assuming ambient pressure is defined as having a value of 1, a vacuum is applied to a first 30 reaction chamber, which may be written $1-3x$, where x is an incremental pressure differential. A vacuum of $1-2x$ is applied to a second reaction chamber in the series, and a vacuum of $1-x$ is applied to a third reaction chamber. Thus, the first reaction chamber is at the lowest pressure and the 35 third is at the highest, with the second being at an intermediate level. All chambers, however, are below ambient pressure, e.g., atmospheric. The sample is drawn into the

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first reaction chamber by the pressure differential between ambient pressure (1) and the vacuum applied to the reaction chamber (1-3x), which differential is -3x. The sample does not move to the second reaction chamber due to the pressure differential between the first and second reaction chambers (1-3x vs. 1-2x, respectively). Upon completion of the operation performed in the first reaction chamber, the vacuum is removed from the first chamber, allowing the first chamber to come to ambient pressure, e.g., 1. The sample is then drawn from the first chamber into the second by the pressure difference between the ambient pressure of the first reaction chamber and the vacuum of the second chamber, e.g., 1 vs. 1-2x. Similarly, when the operation to be performed in the second reaction chamber is completed, the vacuum to this chamber is removed and the sample moves to the third reaction chamber.

A schematic representation of a pneumatic manifold configuration for carrying out this pressure differential fluid transport system is shown in Figure 6A. The pneumatic manifold includes a vacuum source 602 which is coupled to a main vacuum channel 604. The main vacuum channel is connected to branch channels 606, 608 and 610, which are in turn connected to reaction chambers 612, 614 and 616, respectively, which reaction chambers are fluidly connected, in series. The first reaction chamber in the series 616 typically includes a sample inlet 640 which will typically include a sealable closure for retaining the fluid sample and the pressure within the reaction chamber. Each branch channel is provided with one or more fluidic resistors 618 and 620 incorporated within the branch channel. These fluidic resistors result in a transformation of the pressure from the pressure/vacuum source, i.e., a step down of the gas pressure or vacuum being applied across the resistance. Fluidic resistors may employ a variety of different structures. For example, a narrowing of the diameter or cross-sectional area of a channel will typically result in a fluidic resistance through the channel. Similarly, a plug within the channel which has one or more

holes disposed therethrough, which effectively narrow the channel through which the pressure is applied, will result in a fluidic resistance, which resistance can be varied depending upon the number and/or size of the holes in the plug.

5 Additionally, the plug may be fabricated from a porous material which provides a fluidic resistance through the plug, which resistance may be varied depending upon the porosity of the material and/or the number of plugs used. Variations in channel length can also be used to vary fluidic resistance.

10 Each branch channel will typically be connected at a pressure node 622 to the reaction chamber via pressure inlets 624. Pressure inlets 624 will typically be fitted with poorly wetting filter plugs 626, to prevent drawing of the sample into the pneumatic manifold in the case of vacuum based

15 methods. Poorly wetting filter plugs may generally be prepared from a variety of materials known in the art and as described above. Each branch channel is connected to a vent channel 628 which is opened to ambient pressure via vent 630. A differential fluidic resistor 632 is incorporated into vent 20 channel 628. The fluidic resistance supplied by fluidic resistor 632 will be less than fluidic resistance supplied by fluidic resistor 634 which will be less than fluidic resistance supplied by fluidic resistor 636. As described above, this differential fluidic resistance may be 25 accomplished by varying the diameter of the vent channel, varying the number of channels included in a single vent channel, varying channel length, or providing a plug in the vent channel having a varied number of holes disposed therethrough.

30 The varied fluidic resistances for each vent channel will result in a varied level of vacuum being applied to each reaction chamber, where, as described above, reaction chamber 616 may have a pressure of 1-3x, reaction chamber 614 may have a pressure of 1-2x and reaction chamber 612 may have a 35 pressure of 1-x. The pressure of a given reaction chamber may be raised to ambient pressure, thus allowing the drawing of the sample into the subsequent chamber, by opening the chamber

to ambient pressure. This is typically accomplished by providing a sealable opening 638 to ambient pressure in the branch channel. This sealable opening may be a controllable valve structure, or alternatively, a rupture membrane which 5 may be pierced at a desired time to allow the particular reaction chamber to achieve ambient pressure, thereby allowing the sample to be drawn into the subsequent chamber. Piercing of the rupture membrane may be carried out by the inclusion of solenoid operated pins incorporated within the device, or the 10 device's base unit (discussed in greater detail below). In some cases, it may be desirable to prevent back flow from a previous or subsequent reaction chamber which is at a higher pressure. This may be accomplished by equipping the fluid channels between the reaction chambers 644 with one-way check 15 valves. Examples of one-way valve structures include ball and seat structures, flap valves, duck billed check valves, sliding valve structures, and the like.

A graphical illustration of the pressure profiles between three reaction chambers employing a vacuum based 20 pneumatic manifold is shown in Figure 6C. The solid line indicates the starting pressure of each reaction chamber/pressure node. The dotted line indicates the pressure profile during operation. The piercing of a rupture membrane results in an increase in the pressure of the reaction chamber 25 to ambient pressure, resulting in a pressure drop being created between the particular chamber and the subsequent chamber. This pressure drop draws the sample from the first reaction chamber to the subsequent reaction chamber.

In a similar aspect, a positive pressure source may 30 be applied to the originating chamber to push the sample into subsequent chambers. A pneumatic pressure manifold useful in this regard is shown in Figure 6B. In this aspect, a pressure source 646 provides a positive pressure to the main channel 604. Before a sample is introduced to the first reaction 35 chamber, controllable valve 648 is opened to vent the pressure from the pressure source and allow the first reaction chamber in the series 650 to remain at ambient pressure for the

introduction of the sample. Again, the first chamber in the series typically includes a sample inlet 640 having a sealable closure 642. After the sample is introduced into the first reaction chamber 650, controllable valve 648 is closed,
5 bringing the system up to pressure. Suitable controllable valves include any number of a variety of commercially available solenoid valves and the like. In this application, each subsequent chamber is kept at an incrementally higher pressure by the presence of the appropriate fluidic resistors
10 and vents, as described above. A base pressure is applied at originating pressure node 652. When it is desired to deliver the sample to the second chamber 654, sealable opening 656 is opened to ambient pressure. This allows second chamber 654, to come to ambient pressure, allowing the pressure applied at
15 the origin pressure node 652 to force the sample into the second chamber 654. Thus, illustrated as above, the first reaction chamber 650 is maintained at a pressure of $1+x$, by application of this pressure at originating pressure node 652. The second reaction chamber 654 is maintained at pressure $1+2x$
20 and the third reaction chamber 658 is maintained at a pressure of $1+3x$. Opening sealable valve 656 results in a drop in the pressure of the second reaction chamber 654 to 1 (or ambient pressure). The pressure differential from the first to the second reaction chamber, x , pushes the sample from the first
25 to the second reaction chamber and eventually to the third. Fluidic resistor 660 is provided between pressure node 662 and sealable valve 656 to prevent the escape of excess pressure when sealable valve 656 is opened. This allows the system to maintain a positive pressure behind the sample to push it into
30 subsequent chambers.

In a related aspect, a controllable pressure source may be applied to the originating reaction vessel to push a sample through the device. The pressure source is applied intermittently, as needed to move the sample from chamber to
35 chamber. A variety of devices may be employed in applying an intermittent pressure to the originating reaction chamber, e.g., a syringe or other positive displacement pump, or the

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like. Alternatively, for the size scale of the device, a thermopneumatic pump may be readily employed. An example of such a pump typically includes a heating element, e.g., a small scale resistive heater disposed in a pressure chamber.

5 Also disposed in the chamber is a quantity of a controlled vapor pressure fluid, such as a fluorinated hydrocarbon liquid, e.g., fluorinert liquids available from 3M Corp. These liquids are commercially available having a wide range of available vapor pressures. An increase in the controllable

10 temperature of the heater increases pressure in the pressure chamber, which is fluidly connected to the originating reaction chamber. This increase in pressure results in a movement of the sample from one reaction chamber to the next. When the sample reaches the subsequent reaction chamber, the

15 temperature in the pressure chamber is reduced.

The inclusion of gas permeable fluid barriers, e.g., poorly-wetting filter plugs or hydrophobic membranes, in these devices also permits a sensorless fluid direction and control system for moving fluids within the device. For example, as

20 described above, such filter plugs, incorporated at the end of a reaction chamber opposite a fluid inlet will allow air or other gas present in the reaction chamber to be expelled during introduction of the fluid component into the chamber. Upon filling of the chamber, the fluid sample will contact the

25 hydrophobic plug thus stopping net fluid flow. Fluidic resistances, as described previously, may also be employed as gas permeable fluid barriers, to accomplish this same result, e.g., using fluid passages that are sufficiently narrow as to provide an excessive fluid resistance, thereby effectively

30 stopping or retarding fluid flow while permitting air or gas flow. Expelling the fluid from the chamber then involves applying a positive pressure at the plugged vent. This permits chambers which may be filled with no valve at the inlet, i.e., to control fluid flow into the chamber. In most

35 aspects however, a single valve will be employed at the chamber inlet in order to ensure retention of the fluid sample within the chamber, or to provide a mechanism for directing a

fluid sample to one chamber of a number of chambers connected to a common channel.

A schematic representation of a reaction chamber employing this system is shown in Figure 12A. In brief, the 5 reaction chamber 1202 includes a fluid inlet 1204 which is sealed from a fluid passage 1206 by a valve 1208. Typically, this valve can employ a variety of structures, as described herein, but is preferably a flexible diaphragm type valve which may be displaced pneumatically, magnetically or 10 electrically. In preferred aspects, the valves are controlled pneumatically, e.g., by applying a vacuum to the valve to deflect the diaphragm away from the valve seat, thereby creating an opening into adjoining passages. At the end opposite from the inlet, is an outlet vent 1210, and disposed 15 across this outlet vent is a hydrophobic membrane 1212. A number of different commercially available hydrophobic membranes may be used as described herein, including, e.g., Versapore 200 R membranes available from Gelman Sciences. Fluid introduced into the reaction chamber fills the chamber 20 until it contacts the membrane 1212. Closure of the valve then allows performance of reactions within the reaction chamber without influencing or influence from elements outside 25 of the chamber.

In another example, these plugs or membranes may be 25 used for degassing or debubbling fluids within the device. For degassing purposes, for example, a chamber may be provided with one or more vents or with one wall completely or substantially bounded by a hydrophobic membrane to allow the passage of dissolved or trapped gases. Additionally, vacuum 30 may be applied on the external surface of the membrane to draw gases from the sample fluids. Due to the small cross sectional dimensions of reaction chambers and fluid passages, elimination of such gases takes on greater importance, as bubbles may interfere with fluid flow, and/or result in 35 production of irregular data.

In a related aspect, such membranes may be used for removing bubbles purposely introduced into the device, i.e.,

for the purpose of mixing two fluids which were previously desired to be separated. For example, discrete fluids, e.g., reagents, may be introduced into a single channel or debubbling chamber, separated by a gas bubble which is 5 sufficient to separate the fluid plugs but not to inhibit fluid flow. These fluid plugs may then be flowed along a channel having a vent disposed therein, which vent includes a hydrophobic membrane. As the fluid plugs flow past the membrane, the gas will be expelled across the membrane 10 whereupon the two fluids will mix. A schematic illustration of such a debubbling chamber is shown in Figure 12B.

Figure 12C shows a schematic illustration of a device employing a fluid flow system which utilizes hydrophobic membrane bound vents for control of fluid flow. 15 As shown, the device 1250 includes a main channel 1252. The main channel is fluidly connected to a series of separate chambers 1254-1260. Each of these fluid connections with the main channel 1252 is mediated (opened or closed) by the inclusion of a separate valve 1262-1268, respectively, at the 20 intersection of these fluid connections with the main channel. Further, each of the various chambers will typically include a vent port 1270-1276 to the outside environment, which vent ports will typically be bounded by a hydrophobic or poorly wetting membrane. The basic design of this system is 25 reflected in the device schematic shown in Figure 5, as well, in that it employs a central distribution chamber or channel.

In operation, samples or other fluids may be introduced into the main channel 1252 via a valved or otherwise sealable liquid inlet 1278 or 1280. Application of 30 a positive pressure to the fluid inlet, combined with the selective opening of the elastomeric valve at the fluid connection of a selected chamber with the main channel will force the fluid into that chamber, expelling air or other gases through the vent port at the terminus of the selected 35 chamber, until that vent is contacted with the fluid, whereupon fluid flow is stopped. The valve to the selected chamber may then be returned to the closed position to seal

the fluid within the chamber. As described above, the requisite pressure differential needed for fluid flow may alternatively or additionally involve the application of a negative pressure at the vent port to which fluid direction is sought.

As a specific example incorporating the device shown in Figure 12C, a sample introduced into the main channel 1252, is first forced into the degassing chamber 1254 by opening valve 1262 and applying a positive pressure at inlet port 1278. Once the fluid has filled the degassing chamber, valve 1262 may then be closed. Degassing of the fluid may then be carried out by drawing a vacuum on the sample through the hydrophobic membrane disposed across the vent port 1270. Degassed sample may then be moved from the degassing chamber 1254 to, e.g., reaction chamber 1256, by opening valves 1262 and 1264, and applying a positive pressure to the degassing chamber vent port 1270. The fluid is then forced from the degassing chamber 1254, through main channel 1252, into reaction chamber 1256. When the fluid fills the reaction chamber, it will contact the hydrophobic membrane, thereby arresting fluid flow. As shown, the device includes a volumetric or measuring chamber 1258 as well as a storage chamber 1260, including similar valve:vent port arrangements 1266:1274 and 1268:1276, respectively. The fluid may then be selectively directed to other chambers as described.

Figure 12D shows a top view of a portion of an injection molded substrate for carrying out the operations schematically illustrated in Figure 12C. As shown, this device includes liquid loading chambers 1278a and 1280a which are in fluid communication with the fluid inlets 1278 and 1280 (not shown). These fluid inlets may typically be fabricated into the injection molded portion, e.g., drilled into the loading chamber, or fabricated into an overlaying planar member (not shown). Also included are reaction chambers 1254, degassing chambers 1256 and 1256a, measuring chambers 1258, and storage chambers 1260. Each of these chambers is fluidly connected to main channel 1252.

A number of the operations performed by the various reaction chambers of the device require a controllable temperature. For example, PCR amplification, as described above, requires cycling of the sample among a strand separation temperature, an annealing reaction temperature and an extension reaction temperature. A number of other reactions, including extension, transcription and hybridization reactions are also generally carried out at optimized, controlled temperatures. Temperature control within the device of the invention is generally supplied by thin film resistive heaters which are prepared using methods that are well known in the art. For example, these heaters may be fabricated from thin metal films applied within or adjacent to a reaction chamber using well known methods such as sputtering, controlled vapor deposition and the like. The thin film heater will typically be electrically connected to a power source which delivers a current across the heater. The electrical connections will also be fabricated using methods similar to those described for the heaters.

20 Typically, these heaters will be capable of
producing temperatures in excess of 100 degrees without
suffering adverse effects as a result of the heating.
- Examples of resistor heaters include, e.g., the heater
discussed in Published PCT Application No. WO 94\05414,
25 laminated thin film NiCr/polyimide/copper heaters, as well as
graphite heaters. These heaters may be provided as a layer on
one surface of a reaction chamber, or may be provided as
molded or machined inserts for incorporation into the reaction
chambers. Figure 2B illustrates an example of a reaction
30 chamber 104 having a heater insert 128, disposed therein. The
resistive heater is typically electrically connected to a
controlled power source for applying a current across the
heater. Control of the power source is typically carried out
by an appropriately programmed computer. The above-described
35 heaters may be incorporated within the individual reaction
chambers by depositing a resistive metal film or insert within
the reaction chamber, or alternatively, may be applied to the

exterior of the device, adjacent to the particular reaction chamber, whereby the heat from the heater is conducted into the reaction chamber.

Temperature controlled reaction chambers will also typically include a miniature temperature sensor for monitoring the temperature of the chamber, and thereby controlling the application of current across the heater. A wide variety of microsensors are available for determining temperatures, including, e.g., thermocouples having a bimetallic junction which produces a temperature dependent electromotive force (EMF), resistance thermometers which include material having an electrical resistance proportional to the temperature of the material, thermistors, IC temperature sensors, quartz thermometers and the like. See, Horowitz and Hill, *The Art of Electronics*, Cambridge University Press 1994 (2nd Ed. 1994). One heater/sensor design that is particularly suited to the device of the present invention is described in, e.g., U.S. Patent Application Serial No. 08/535,875, filed September 28, 1995, and incorporated herein by reference in its entirety for all purposes. Control of reaction parameters within the reaction chamber, e.g., temperature, may be carried out manually, but is preferably controlled via an appropriately programmed computer. In particular, the temperature measured by the temperature sensor and the input for the power source will typically be interfaced with a computer which is programmed to receive and record this data, i.e., via an analog-digital/digital-analog (AD/DA) converter. The same computer will typically include programming for instructing the delivery of appropriate current for raising and lowering the temperature of the reaction chamber. For example, the computer may be programmed to take the reaction chamber through any number of predetermined time/temperature profiles, e.g., thermal cycling for PCR, and the like. Given the size of the devices of the invention, cooling of the reaction chambers will typically occur through exposure to ambient temperature, however additional cooling elements may be

included if desired, e.g., coolant systems, peltier coolers, water baths, etc.

In addition to fluid transport and temperature control elements, one or more of the reaction chambers of the device may also incorporate a mixing function. For a number of reaction chambers, mixing may be applied merely by pumping the sample back and forth into and out of a particular reaction chamber. However, in some cases constant mixing within a single reaction/analytical chamber is desired, e.g., PCR amplification reactions and hybridization reactions.

In preferred aspects, acoustic mixing is used to mix the sample within a given reaction chamber. In particular, a PZT element (element composed of lead, zirconium and titanium containing ceramic) is contacted with the exterior surface of the device, adjacent to the reaction chamber, as shown in Figure 7A. For a discussion of PZT elements for use in acoustic based methods, see, *Physical Acoustics, Principles and Methods*, Vol. I, (Mason ed., Academic Press, 1965), and *Piezoelectric Technology, Data for Engineers*, available from Clevite Corp. As shown, PZT element 702 is contacting the external surface 704 of hybridization chamber 706. The hybridization chamber includes as one internal surface, an oligonucleotide array 708. Application of a current to this element generates sonic vibrations which are translated to the reaction chamber whereupon mixing of the sample disposed therein occurs. The vibrations of this element result in substantial convection being generated within the reaction chamber. A symmetric mixing pattern generated within a micro reaction chamber incorporating this mixing system is shown Figure 7B.

Incomplete contact (i.e., bonding) of the element to the device may result in an incomplete mixing of a fluid sample. As a result, the element will typically have a fluid or gel layer (not shown) disposed between the element 702 and the external surface of the device 704, e.g., water. This fluid layer will generally be incorporated within a membrane, e.g., a latex balloon, having one surface in contact with the

external surface of the reaction chamber and another surface in contact with the PZT element. An appropriately programmed computer 714 may be used to control the application of a voltage to the PZT element, via a function generator 712 and 5 RF amplifier 710 to control the rate and/or timing of mixing.

In alternate aspects, mixing may be supplied by the incorporation of ferromagnetic elements within the device which may be vibrated by supplying an alternating current to a coil adjacent the device. The oscillating current creates an 10 oscillating magnetic field through the center of the coil which results in vibratory motion and rotation of the magnetic particles in the device, resulting in mixing, either by direct convection or accoustic streaming.

In addition to the above elements, the devices of 15 the present invention may include additional components for optimizing sample preparation or analysis. For example, electrophoretic force may be used to draw target molecules into the surface of the array. For example, electrodes may be disposed or patterned on the surface of the array or on the 20 surface opposite the array. Application of an appropriate electric field will either push or pull the targets in solution onto the array. A variety of similar enhancements can be included without departing from the scope of the invention.

25 Although it may often be desirable to incorporate all of the above described elements within a single disposable unit, generally, the cost of some of these elements and materials from which they are fabricated, may make it desirable to provide a unit that is at least partially reusable. Accordingly, in a particularly preferred 30 embodiment, a variety of control elements for the device, e.g., temperature control, mixing and fluid transport elements may be supplied within a reusable base-unit.

For example, in a particularly preferred embodiment, 35 the reaction chamber portion of the device can be mated with a reusable base unit that is adapted for receiving the device. As described, the base unit may include one or more heaters

for controlling the temperature within selected reaction chambers within the device. Similarly, the base unit may incorporate mixing elements such as those described herein, as well as vacuum or pressure sources for providing sample mixing 5 and transportation within the device.

As an example, the base unit may include a first surface having disposed thereon, one or more resistive heaters of the type described above. The heaters are positioned on the surface of the base unit such that when the reaction 10 chamber device is mated to that surface, the heaters will be adjacent to and preferably contacting the exterior surface of the device adjacent to one or more reaction chambers in which temperature control is desired. Similarly, one or more mixing elements, such as the acoustic mixing elements described 15 above, may also be disposed upon this surface of the base unit, whereby when mated with the reaction chamber device, the mixing elements contact the outer surface of the reaction/storage/analytical chambers in which such mixing is desired. For those reaction chambers in which both mixing and 20 heating are desired, interspersed heaters and mixers may be provided on the surface of the base unit. Alternatively, the base unit may include a second surface which contacts the opposite surface of the device from the first surface, to apply heating on one exterior surface of the reaction chamber 25 and mixing at the other.

Along with the various above-described elements, the base unit also typically includes appropriate electrical connections for linking the heating and mixing elements to an appropriate power source. Similarly, the base unit may also 30 be used to connect the reaction chamber device itself to external power sources, pressure/vacuum sources and the like. In particular, the base unit can provide manifolds, ports and electrical connections which plug into receiving connectors or 35 ports on the device to provide power, vacuum or pressure for the various control elements that are internal to the device. For example, mating of the device to the base unit may provide a connection from a vacuum source in the base unit to a main

vacuum manifold manufactured into the device, as described above. Similarly, the base unit may provide electrical connectors which couple to complementary connectors on the device to provide electrical current to any number of 5 operations within the device via electrical circuitry fabricated into the device. Similarly, appropriate connections are also provided for monitoring various operations of the device, e.g., temperature, pressure and the like.

10 For those embodiments employing a pneumatic manifold for fluid transport which relies on the piercing of rupture membranes within the device to move the sample to subsequent chambers, the base unit will also typically include one or more solenoid mounted rupture pins. The solenoid mounted 15 rupture pins are disposed within receptacles which are manufactured into the surface of the base unit, which receptacles correspond to positions of the rupture membranes upon the device. The pins are retained below the surface of the base unit when not in operation. Activation of the 20 solenoid extends the pin above the surface of the base unit, into and through the rupture membrane.

A schematic representation of one embodiment of a base unit is shown in Figure 8. As shown in Figure 8, the base unit 800 includes a body structure 802 having a mating 25 surface 804. The body structure houses the various elements that are to be incorporated into the base unit. The base unit may also include one or more thermoelectric heating/cooling elements 806 disposed within the base unit such that when the reaction chamber containing portion of the apparatus is mated 30 to the mating surface of the base unit, the reaction chambers will be in contact or immediately adjacent to the heating elements. For those embodiments employing a differential pressure based system for moving fluids within the device, as described above, the base unit may typically include a 35 pressure source opening to the mating surface via the pressure source port 810. The base unit will also typically include other elements of these systems, such as solenoid 812 driven

pins 814 for piercing rupture membranes. These pins are typically within recessed ports 816 in the mating surface 804. The base unit will also typically include mounting structures on the mating surface to ensure proper mating of the reaction 5 chamber containing portion of the device to the base unit. Such mounting structures generally include mounting pins or holes (not shown) disposed on the mating surface which correspond to complementary structures on the reaction chamber containing portion of the device. Mounting pins may be 10 differentially sized, and/or tapered, to ensure mating of the reaction chamber and base unit in an appropriate orientation. Alternatively, the base unit may be fabricated to include a well in which the reaction chamber portion mounts, which well has a nonsymmetrical shape, matching a nonsymmetrical shape of 15 the reaction chamber portion. Such a design is similar to that used in the manufacture of audio tape cassettes and players.

In addition to the above described components, the device of the present invention may include a number of other 20 components to further facilitate analyses. In particular, a number of the operations of sample transport, manipulation and monitoring may be performed by elements external to the device, per se. These elements may be incorporated within the above-described base unit, or may be included as further 25 attachments to the device and/or base unit. For example, external pumps or fluid flow devices may be used to move the sample through the various operations of the device and/or for mixing, temperature controls may be applied externally to the device to maximize individual operations, and valve controls 30 may be operated externally to direct and regulate the flow of the sample. In preferred embodiments, however, these various operations will be integrated within the device. Thus, in addition to the above described components, the integrated device of the invention will typically incorporate a number of 35 additional components for sample transporting, direction, manipulation, and the like. Generally, this will include a plurality of micropumps, valves, mixers and heating elements.

Pumping devices that are particularly useful include a variety of micromachined pumps that have been reported in the art. For example, suitable pumps include pumps which having a bulging diaphragm, powered by a piezoelectric stack 5 and two check valves, such as those described in U.S. Patent Nos. 5,277,556, 5,271,724 and 5,171,132, or powered by a thermopneumatic element, as described in U.S. Patent No. 5,126,022 piezoelectric peristaltic pumps using multiple membranes in series, and the like. The disclosure of each of 10 these patents is incorporated herein by reference. Published PCT Application No. WO 94/05414 also discusses the use of a lamb-wave pump for transportation of fluid in micron scale channels.

Ferrofluidic fluid transport and mixing systems may 15 also be incorporated into the device of the present invention. Typically, these systems incorporate a ferrofluidic substance which is placed into the apparatus. The ferrofluidic substance is controlled/directed externally through the use of magnets. In particular, the ferrofluidic substance provides a 20 barrier which can be selectively moved to force the sample fluid through the apparatus, or through an individual operation of the apparatus. These ferrofluidic systems may be used for example, to reduce effective volumes where the sample 25 occupies insufficient volume to fill the hybridization chamber. Insufficient sample fluid volume may result in incomplete hybridization with the array, and incomplete hybridization data. The ferrofluidic system is used to sandwich the sample fluid in a sufficiently small volume. This small volume is then drawn across the array in a manner 30 which ensures the sample contacts the entire surface of the array. Ferrofluids are generally commercially available from, e.g., FerroFluidics Inc., New Hampshire.

Alternative fluid transport mechanisms for inclusion within the device of the present invention include, e.g. 35 electrohydrodynamic pumps (see, e.g., Richter, et al. 3rd IEEE Workshop on Micro Electro Mechanical Systems, February 12-14, 1990, Napa Valley, USA, and Richter et al., Sensors and

Actuators 29:159-165 (1991), U.S. Patent No. 5,126,022, each of which is incorporated herein by reference in its entirety for all purposes). Typically, such pumps employ a series of electrodes disposed across one surface of a channel or 5 reaction/pumping chamber. Application of an electric field across the electrodes results in electrophoretic movement of nucleic acids in the sample. Indium-tin oxide films may be particularly suited for patterning electrodes on substrate surfaces, e.g., a glass or silicon substrate. These methods 10 can also be used to draw nucleic acids onto an array. For example, electrodes may be patterned on the surface of an array substrate and modified with suitable functional groups for coupling nucleic acids to the surface of the electrodes. Application of a current between the electrodes on the surface 15 of an array and an opposing electrode results in electrophoretic movement of the nucleic acids toward the surface of the array.

Electrophoretic pumping by application of transient electric fields can also be employed to avoid electrolysis at 20 the surface of the electrodes while still causing sufficient sample movement. In particular, the electrophoretic mobility of a nucleic acid is not constant with the electric field applied. An increase in an electric field of from 50 to 400 v/cm results in a 30% increase in mobility of a nucleic acid 25 sample in an acrylamide gel. By applying an oscillating voltage between a pair of electrodes capacitively coupled to the electrolyte, a net electrophoretic motion can be obtained without a net passage of charge. For example, a high electric field is applied in the forward direction of sample movement 30 and a lower field is then applied in the reverse direction. See, e.g., Luckey, et al., Electrophoresis 14:492-501 (1993).

The above described micropumps may also be used to mix reagents and samples within the apparatus, by directing a recirculating fluid flow through the particular chamber to be 35 mixed. Additional mixing methods may also be employed. For example, electrohydrodynamic mixers may be employed within the various reaction chambers. These mixers typically employ a

traveling electric field for moving a fluid into which a charge has been introduced. See Bart, et al., *Sensors and Actuators* (1990) A21-A-23:193-197. These mixing elements can be readily incorporated into miniaturized devices.

5 Alternatively, mixing may be carried out using thermopneumatic pumping mechanism. This typically involves the inclusion of small heaters, disposed behind apertures within a particular chamber. When the liquid in contact with the heater is heated, it expands through the apertures causing a convective 10 force to be introduced into the chamber, thereby mixing the sample. Alternatively, a pumping mechanism retained behind two one way check valves, such as the pump described in U.S. Patent No. 5,375,979 to Trah, incorporated herein by reference in its entirety for all purposes, can be employed to circulate 15 a fluid sample within a chamber. In particular, the fluid is drawn into the pumping chamber through a first one-way check valve when the pump is operated in its vacuum or drawing cycle. The fluid is then expelled from the pump chamber through another one way check valve during the reciprocal pump 20 cycle, resulting in a circular fluid flow within the reaction chamber. The pumping mechanism may employ any number of designs, as described herein, i.e., diaphragm, thermal pressure, electrohydrodynamic, etc.

It will typically be desirable to insulate 25 electrical components of the device which may contact fluid samples, to prevent electrolysis of the sample at the surface of the component. Generally, any number of non-conducting insulating materials may be used for this function, including, e.g., teflon coating, SiO_2 , Si_3N_4 , and the like. Preferably, 30 insulating layers will be SiO_2 , which may generally be sputtered over the surface of the component to provide an insulating layer.

The device of the present invention will also typically incorporate a number of microvalves for the 35 direction of fluid flow within the device. A variety of microvalve designs are particularly well suited for the instant device. Examples of valves that may be used in the

device are described in, e.g., U.S. Patent No. 5,277,556 to van Lintel, incorporated herein by reference. Preferred valve structures for use in the present devices typically incorporate a membrane or diaphragm which may be deflected onto a valve seat. For example, the electrostatic valves, silicon/aluminum bimetallic actuated valves or thermopneumatic actuated valves can be readily adapted for incorporation into the device of the invention. Typically, these valves will be incorporated within or at one or both of the termini of the fluid channels linking the various reaction chambers, and will be able to withstand the pressures or reagents used in the various operations. An illustration of an embodiment of the diaphragm valve/fluid channel construction is illustrated in Figure 3.

In alternative aspects, fluidic valves may also be employed. Such fluidic valves typically include a "liquid curtain" which comprises a fluid that is immiscible in the aqueous systems used in the device, e.g., silicone oil, ferrofluidic fluids, and the like. In operation, a fluidic valve includes a shallow valving channel, e.g. 50 μm deep, disposed transversely across and interrupting a deeper primary channel, e.g., a 200 μm deep channel in a mating planar member. The valving channel is connected to at least one oil port. In operation, the valving channel is first filled with oil (or other appropriate fluid element), which is drawn into the channel by capillary action. When gas or liquid are forced through the primary channel, the oil, or "fluid curtain" moves aside and allows passage. In the absence of differential pressure along the primary channel, the oil will return to seal the fluid or gas behind a vapor barrier. In such cases, these fluidic valves are useful in the prevention of evaporation of fluid samples or reagents within the device. Additionally, in the case of other fluids, e.g., ferrofluids or oils with suspended metallic particles, application of an appropriate magnetic field at the valve position immobilizes the fluidic valve, thereby resisting fluid passage at pressures greater than 3-5 psi. Similarly, electrorheological

effects may also be employed in controlling these fluidic valves. For example, the oil portion of the fluid valve may have suspended therein appropriate particles having high dielectric constants. Application of an appropriate electric field then increases the viscosity of the fluid thereby creating an appropriate barrier to fluid flow.

The device may also incorporate one or more filters for removing cell debris and protein solids from the sample. The filters may generally be within the apparatus, e.g., within the fluid passages leading from the sample preparation/extraction chamber. A variety of well known filter media may be incorporated into the device, including, e.g., cellulose, nitrocellulose, polysulfone, nylon, vinyl/acrylic copolymers, glass fiber, polyvinylchloride, and the like. Alternatively, the filter may be a structure fabricated into the device similar to that described in U.S. Patent No. 5,304,487 to Wilding et al., previously incorporated herein. Similarly, separation chambers having a separation media, e.g., ion exchange resin, affinity resin or the like, may be included within the device to eliminate contaminating proteins, etc.

In addition to sensors for monitoring temperature, the device of the present invention may also contain one or more sensors within the device itself to monitor the progress of one or more of the operations of the device. For example, optical sensors and pressure sensors may be incorporated into one or more reaction chambers to monitor the progress of the various reactions, or within flow channels to monitor the progress of fluids or detect characteristics of the fluids, e.g., pH, temperature, fluorescence and the like.

As described previously, reagents used in each operation integrated within the device may be exogenously introduced into the device, e.g., through sealable openings in each respective chamber. However, in preferred aspects, these reagents will be predisposed within the device. For example, these reagents may be disposed within the reaction chamber which performs the operation for which the reagent will be

used, or within the fluid channels leading to that reaction chamber. Alternatively, the reagents may be disposed within storage chambers adjacent to and fluidly connected to their respective reaction chambers, whereby the reagents can be 5 readily transported to the appropriate chamber as needed. For example, the amplification chamber will typically have the appropriate reagents for carrying out the amplification reaction, e.g., primer probe sequences, deoxynucleoside triphosphates ("dNTPs"), nucleic acid polymerases, buffering 10 agents and the like, predisposed within the amplification chamber. Similarly, sample stabilization reagents will typically be predisposed within the sample collection chamber.

2. Generic Sample Preparation Device

Figure 13 shows a schematic illustration of a device 15 configuration for performing sample preparation reactions, generally, utilizing the fluid direction systems described herein, e.g., employing external pressures, hydrophobic vents and pneumatic valves. In the configuration shown, four domains of the device are each addressed by an array of 20 valves, e.g., a 10 valve array, with its own common channel. The four domains may generally be defined as: (1) reagent storage; (2) reaction; (3) sample preparation; and (4) post 25 processing, which are fluidically interconnected. The sample preparation domain is typically used to extract and purify nucleic acids from a sample. As shown, included in the sample preparation domain are 5 reagent inlets that are fluidly connected to larger volume storage vessels, e.g., within the base unit. Examples of such reagents for extraction reactions may include, e.g., 4M guanidine isothiocyanate, 1 X TBE and 30 50:50 ETOH:H₂O. The two reaction chambers may include, e.g., affinity media for purification of nucleic acids such as glass wool, or beads coated with poly-T oligonucleotides.

The storage domain is linked to the sample preparation domain, and is used for storage of reagents and 35 mixtures, e.g., PCR mix with FITC-dGTP and dUTP but no template, UNG reaction mix and IVT reaction mix without template. The reaction domain is also linked to the sample

preparation domain as well as the storage domain and includes a number of reaction chambers (5), measuring chambers (2) and debubbling chambers (1). Both sample preparation and reaction domains may be addressed by a thermal controller, e.g.,
5 heaters or thermoelectric heater/cooler.

The post processing domain is typically linked to the reaction domain and includes a number of reagent inlets (5), reaction chambers (2), storage chambers (1) and sample inlets (1). The reagent inlets may be used to introduce
10 buffers, e.g., 6 X SSPE or water into the analytical element, e.g., an oligonucleotide array.

3. Generic Multiple Parallel System

Figure 14 is a schematic illustration of a device configuration for addressing situations where several reactions
15 are to be carried out under the same thermal conditions, e.g., multiple parallel sample analyses, duplicating multiplex PCR by carrying out several PCR reactions with single primer pairs in parallel followed by recombining them, or cycle sequencing with a variety of primer pairs and/or templates.

20 In this configuration as shown, two storage domains supply reagents to two reaction domains, each being addressed by an array of 50 valves. The reaction and storage arrays each comprise a 4 X 12 matrix of reactors/chambers, each from 10 nl to 5 μ l in volume. These chambers are addressed by 4
25 columns each of pneumatic ports. Two additional arrays of 10 valves address a sample preparation and post processing domain. A bank of solenoid valves may be used to drive the pneumatic ports and the valve arrays.

30 IV. Applications

The device and system of the present invention has a wide variety of uses in the manipulation, identification and/or sequencing of nucleic acid samples. These samples may be derived from plant, animal, viral or bacterial sources.
35 For example, the device and system of the invention may be used in diagnostic applications, such as in diagnosing genetic disorders, as well as diagnosing the presence of infectious

agents, e.g., bacterial or viral infections. Additionally, the device and system may be used in a variety of characterization applications, such as forensic analysis, e.g., genetic fingerprinting, bacterial, plant or viral 5 identification or characterization, e.g., epidemiological or taxonomic analysis, and the like.

Although generally described in terms of individual devices, it will be appreciated that multiple devices may be provided in parallel to perform analyses on a large number of 10 individual samples. because the devices are miniaturized, reagent and/or space requirements are substantially reduced. Similarly, the small size allows automation of sample introduction process using, e.g., robot samplers and the like.

In preferred aspects, the device and system of the 15 present invention is used in the analysis of human samples. More particularly, the device is used to determine the presence or absence of a particular nucleic acid sequence within a particular human sample. This includes the identification of genetic anomalies associated with a 20 particular disorder, as well as the identification within a sample of a particular infectious agent, e.g., virus, bacteria, yeast or fungus.

The devices of the present invention may also be used in de novo sequencing applications. In particular, the 25 device may be used in sequencing by hybridization (SBH) techniques. The use of oligonucleotide arrays in de novo SBH applications is described, for example, in U.S. Application Serial No. 08/082,937, filed June 25, 1993.

30

EXAMPLES

Example 1- Extraction and Purification of Nucleic Acids

In separate experiments, HIV cloned DNA was spiked into either horse blood or a suspension of murine plasmacytoma fully differentiated B-cells derived from BALBc mice. 35 Guanidine isothiocyanate was added to a concentration of 4 M, to lyse the material. In separate experiments, the lysate was passed through a cartridge containing glass wool (20 μ l), a

cartridge with soda glass walls (20 μ l), and a glass tube. After 30 minutes at room temperature, the remaining lysate was washed away with several volumes of ethanol:water (1:1) and the captured DNA was eluted at 60°C using 1X TBE. The yield 5 of eluted DNA was measured using ethidium bromide staining on an agarose gel, and purity was tested by using the eluted material as a template for a PCR reaction. Elution yields ranged from 10% to 25% and PCR yields ranged from 90 to 100% as compared to controls using pure template.

10

Example 2- RNA Preparation Reactions in Miniaturized System

A model miniature reactor system was designed to investigate the efficacy of miniaturized devices in carrying out prehybridization preparative reactions on target nucleic acids. In particular, a dual reaction chamber system for carrying out in vitro transcription and fragmentation was fabricated. The device employed a tube based structure using a polymer tubing as an in vitro transcription reactor coupled to a glass capillary fragmentation reactor. Reagents not introduced with the sample were provided as dried deposits on the internal surface of the connecting tubing. The experiment was designed to investigate the effects of reaction chamber materials and reaction volume in RNA preparative reaction chambers.

The sample including the target nucleic acid, DNA amplicons containing a 1 kb portion of the HIV gene flanked with promoter regions for the T3 and T7 RNA primers on the sense and antisense strands, respectively, RNA polymerase, NTPs, fluorinated UTP and buffer, were introduced into the reactor system at one end of the tubing based system. In vitro transcription was carried out in a silicone tubing reactor immersed in a water bath. Following this initial reaction, the sample was moved through the system into a glass capillary reactor which was maintained at 94°C, for carrying out the fragmentation reaction. The products of a representative time-course fragmentation reaction are shown in the gel of Figure 10A. In some cases, the tubing connecting

the IVT reactor to the fragmentation reactor contained additional $MgCl_2$ for addition to the sample. The glass capillary was first coated with BSA to avoid interactions between the sample and the glass. Following fragmentation, 5 the sample was hybridized with an appropriately tiled oligonucleotide array, as described above. Preparation using this system with 14 mM $MgCl_2$ addition resulted in a correct base calling rate of 96.5%. Omission of the $MgCl_2$ gave a correct base calling rate of 95.5%.

10 A similar preparative transcription reaction was carried out in a micro-reaction chamber fabricated in polycarbonate. A well was machined in the surface of a first polycarbonate part. The well was 250 μm deep and had an approximate volume of 5 μl . A second polycarbonate part was 15 then acoustically welded to the first to provide a top wall for the reaction chamber. The second part had two holes drilled through it, which holes were positioned at opposite ends of the reaction chamber. Temperature control for the transcription reaction was supplied by applying external 20 temperature controls to the reaction chamber, as described for the tubing based system. 3 μl samples were used for both transcription and fragmentation experiments.

Transcription reactions performed in the micro-reactor achieved a 70% yield as compared to conventional 25 methods, e.g., same volume in microfuge tube and water bath or PCR thermal cycler. A comparison of in vitro transcription reaction products using a microchamber versus a larger scale control are shown in Figure 10B.

30 Example 3- PCR Amplification in Miniaturized System

The miniature polymeric reaction chamber similar to the one described in Example 2 was used for carrying out PCR amplification. In particular, the chamber was fabricated from a planar piece of polycarbonate 4 mm thick, and having a cavity 35 measuring 500 μm deep machined into its surface. A second planar polycarbonate piece was welded over the cavity. This second piece was only 250 μm thick. Thermal control was

supplied by applying a peltier heater against the thinner second wall of the cavity.

Amplification of a target nucleic acid was performed with Perkin-Elmer GeneAmp® PCR kit. The reaction chamber was 5 cycled for 20 seconds at 94°C (denaturing), 40 seconds at 65°C (annealing) and 50 seconds at 72°C (extension). A profile of the thermal cycling is shown in Figure 9. Amplification of approximately 10^9 was shown after 35 cycles. Figure 10C shows production of amplified product in the microchamber as 10 compared to a control using a typical PCR thermal cycler.

Example 4- System Demonstration, Integrated Reactions

A microfabricated polycarbonate device was manufactured having the structure shown in Figure 15A. The 15 device included three discrete vented chambers. Two of the chambers (top and middle) were thermally isolated from the PCR chamber (bottom) to prevent any denaturation of the RNA polymerase used in IVT reractions at PCR temperatures. Thermal isolation was accomplished by fabricating the chambers 20 more than 10 mm apart in a thin polycarbonate substrate and controlling the temperatures in each region through the use of thermoelectric temperature controllers, e.g., peltier devices.

The reactor device dimensions were as follows: channels were 250 μm wide by 125 μm deep; the three reaction 25 chambers were 1.5 mm wide by 13 mm in length by 125 to 500 μm deep, with the reactor volumes ranging from 2.5 to 10 μl . Briefly, PCR was carried out by introducing 0.3 units of Taq 30 polymerase, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.2 μM primer sequences, approximately 2000 molecules of template sequence and 1X Perkin-Elmer PCR buffer into the bottom chamber. The thermal cycling program included (1) an initial denaturation 35 at 94°C for 60 seconds, (2) a denaturation step at 94°C for 20 seconds, (3) an annealing step at 65°C for 40 seconds, (4) an extension step at 72°C for 50 seconds, (5) repeated cycling through steps 2-4 35 times, and (6) a final extension step at 72°C for 60 seconds.

Following PCR, 0.2 μ l of the PCR product was transferred to the IVT chamber (middle) along with 9.8 μ l of IVT mixture (2.5 mM ATP, CTP, GTP and 0.5 mM UTP, 0.25 mM Fluorescein-UTP, 8 mM MgCl₂, 50 mM HEPES, 1X Promega 5 Transcription Buffer, 10 mM DTT, 1 unit T3 RNA polymerase, 0.5 units RNAGuard (Pharmacia)) that had been stored in a storage chamber (top). Fluid transfer was carried out by applying pressure to the vents at the termini of the chambers. IVT was carried out at 37°C for 60 minutes.

10 The results of PCR and IVT are shown in Figure 15B, compared with control experiments, e.g., performed in eppendorf tubes.

Example 5- Acoustic Mixing

15 The efficacy of an acoustic element for mixing the contents of a reaction chamber was tested. A 0.5" X 0.5" X 0.04" crystal of PZT-5H was bonded to the external surface of a 0.030" thick region of a planar piece of delrin which had cavity machined in the surface opposite the PZT element. An 20 oligonucleotide array synthesized on a flat silica substrate, was sealed over the cavity using a rubber gasket, such that the surface of the array having the oligonucleotide probes synthesized on it was exposed to the cavity, yielding a 250 μ l reaction chamber. The PZT crystal was driven by an ENI200 25 High Frequency Power Supply, which is driven by a function generator from Hewlett Packard that was gated by a second function generator operated at 1 Hz.

In an initial test, the chamber was filled with deionized water and a small amount of 2% milk was injected for 30 visualization. The crystal was driven at 2 MHz with an average power of 3 W. Fluid velocities within the chamber were estimated in excess of 1 mm/sec, indicating significant convection. A photograph showing this convection is shown in Figure 7B.

35 The efficacy of acoustic mixing was also tested in an actual hybridization protocol. For this hybridization test, a fluorescently labeled oligonucleotide target sequence

having the sequence 5'-GAGATGCCGTCGGTGGCTG-3' and an array having a checkerboard pattern of 400 μm squares having complements to this sequence synthesized thereon, were used. Hybridization of a 10 nM solution of the target in 6xSSPE was 5 carried out. During hybridization, the external surface of the array was kept in contact with a thermoelectric cooler set at 15°C. Hybridization was carried out for 20 minutes while driving the crystal at 2 MHz at an average power of 4 W (on time = 0.2 sec., off time = 0.8 sec.). The resulting average 10 intensity was identical to that achieved using mechanical mixing of the chamber (vertical rotation with an incorporated bubble).

Additional experiments using fluorescently labeled and fragmented 1 kb portion of the HIV virus had a successful 15 base calling rates. In particular, a 1 kb HIV nucleic acid segment was sequenced using an HIV tiled oligonucleotide array or chip. See, U.S. Patent Application Serial No. 08/284,064, filed August 2, 1994, and incorporated herein by reference for all purposes. Acoustic mixing achieved a 90.5% correct base 20 calling rate as compared to a 95.8% correct base calling rate for mechanical mixing.

Example 5- Demonstration of Fluid Direction System

A polycarbonate cartridge was fabricated using 25 conventional machining, forming an array of valves linking a common channel to a series of channels leading to a series of 10 μl chambers, each of which was terminated in a hydrophobic vent. The chambers included (1) an inlet chamber #1, (2) inlet chamber #2, (3) reaction chamber, (4) debubbling chamber 30 having a hydrophobic vent in the center, (5) a measuring chamber and (6) a storage chamber. Elastomeric valves were opened and closed by application of vacuum or pressure (approx. 60 psi) to the space above the individual valves.

In a first experiment, water containing blue dye 35 (food coloring) was introduced into inlet chamber #1 while water containing yellow dye (food coloring) was introduced into inlet chamber #2. By opening the appropriate valves and

applying 5 psi to the appropriate vent, the following series of fluid movements were carried out: the blue water was moved from inlet chamber #1 to the reaction chamber; the yellow water was moved from inlet chamber #2 to the storage chamber # 5 6; the blue water was moved from the reaction chamber to the measuring chamber and the remaining blue water was exhausted to the inlet chamber #1; The measured blue water (approximately 1.6 μ l) was moved from the measuring chamber to the debubbling chamber; the yellow water is then moved from 10 the storage chamber into the debubbling chamber whereupon it linked with the blue water and appeared to mix, producing a green color; and finally, the mixture was moved from the debubbling chamber to the reaction chamber and then to the storage chamber.

15 Functioning of the debubbling chamber was demonstrated by moving four separate plugs of colored water from the reaction chamber to the debubbling chamber. The discrete plugs, upon passing into the debubbling chamber, joined together as a single fluid plug.

20 The functioning of the measuring chamber was demonstrated by repetetively moving portions of a 10 μ l colored water sample from the storage chamber to the measuring chamber, followed by exhausting this fluid from the measuring chamber. This fluid transfer was carried out 6 times, 25 indicating repeated aliquoting of approximately 1.6 μ l per measuring chamber volume (10 μ l in 6 aliquots).

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this 30 disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication 35 or patent document were so individually denoted.